Effect of manipulation of the renin-angiotensin system on the osmoregulatory responses of silver seabream (*Sparus sarba*) in hyper- and hypo-osmotic media

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Abstract

There are extensive studies on the role of the Renin-angiotensin system (RAS) in teleostean osmoregulation. Angiotensin (Ang) is an active pressor substance and RAS is responsible for the regulation of blood pressure (BP) and blood volume (BV) in vertebrates. Volume depletion, hypotension, and any other challenges that induce similar effects will stimulate the RAS to increase the BP. Both seawater (SW) and freshwater (FW) fishes are subjected to osmoregulatory challenges. SW fishes experience hypovolemia as they lose water to the external environment and this triggers the activation of RAS to elevate BP. Studies also showed that angiotensin is a dipsogen in vertebrates and stimulated water intake in most teleosts, both in FW and SW. Various other effects including anti-diuretic, increase in plasma cortisol level, elevation of brachial and renal Na^+/K^+-ATPase activity, stimulation of pituitary secretion of adrenocorticotrophic hormone (ACTH), are also known to be controlled by RAS.

Silver Seabream (*S. sarba*) is a SW fish but has been found to survive in a wide range of salinities (6 ppt – 50 ppt). Physiological parameters including BP, BV, and drinking rate, are assessed for the study of a possible contribution of RAS to the euryhalinity of *S. sarba*. Brachial angiotensin converting enzyme (ACE) of different salinity-adapted fish is investigated to show the status of RAS of the fish in particular

salinity. Characteristics of oxygen dissociation and hemoglobin are also examined to study the effects of salinity on different aspects of body organization.

The BV of seabream is tightly controlled in various situations, including long term salinity adaptation and short term salinity challenge. The oxygen dissociation curve in various salinities showed high level of similarities and hemoglobin analysis also revealed that same types of isoforms have been produced in different salinities. Abrupt transfer of fish from 33 ppt to 6 ppt or *vice versa*, does not change the blood pressure of the fish. Injection of captopril, which is a potent inhibitor of ACE, lowers the basal BP of both 33 and 6 ppt fish, showing RAS is responsible for basal BP regulation in seabream. Abrupt osmotic challenge from 33 ppt to 6 ppt or *vice versa* after captopril treatment, also does not affect the BP, indicating the BP homeostasis is not controlled exclusively by RAS. Drinking rate of the hyposmotic-adapted fish is lower than that of hyperosmotic-adapted fish. Injection of captopril, and abrupt transfer of fish from 33 ppt to 6 ppt, lower the drinking rate of SW fish, showing that RAS plays an important role in drinking rate regulation. Injection of angiotensin elevates the drinking rate of hyposmotic-adapted fish, but the Ang treatments lowered the drinking rate in hyperosmotic-adapted fish. Activation of endogenous RAS by vasodilator sodium nitroprusside (SNP), lowered the drinking rate in hyperosmotic-adapted fish but elevated the drinking rate in

hyposmotic-adapted one. Possible involvement of RAS and kallikrein-kinin system (KKS) in the regulation of BP and drinking rate in respect to the bimodal relationship with Ang in seabream is discussed.

摘要

血管緊張素是一種升壓物質。腎素-血管緊張素系統負責控制脊椎類動物的血壓和血容量。血容量下降，低血壓，或其他類似的情況也能刺激腎素-血管緊張素系統從而導致血壓上升。海水和淡水魚類承受著不同的滲透壓。因爲海水的滲透壓高於魚血的滲透壓，而導致體液滲透流失,所以海水魚類的血容量下降，從而刺激腎素-血管緊張素系統而導致血壓回升。研究發現血管緊張素可增加脊椎類動物的飲水量。對魚類來說，無論是海水或淡水，血管緊張素也可以提高牠們的飲水量，同時腎素-血管緊張素系統又能影響著魚類的其他生理反應。例如它能抗利尿，增加血液中的皮質醇，提高鰓和腎的鈉-鉀-三磷酸腺甘酶的活性，並刺激腦下垂體分泌促腎上腺皮質激素等。

雖然鯛魚被認爲是海水魚類，但是牠能於不同鹽度的水裏生存。本文通過一些生理參數包括血壓，血容量和飲水量來研究腎素-血管緊張素系統對鯛魚的廣鹽性的重要性。不同鹽度下生活的魚鰓裏的血管緊張素轉化酶活性，可以顯示魚類在特定鹽度下腎素-血管緊張素系統的狀況。本研究也指出不同鹽度怎樣影響魚類的血紅素和氧解離曲線特徵的關係。

無論是長期或短期鹽度適應，鯛魚的血容量也是被緊密控制而達至平衡。在不同鹽度裏的氧解離曲線非常相似，而血紅素分析也指出在不同鹽度生活的鯛魚會製造相同的同血紅素。無論由鹽度千分之三十三突然降至千分之六或相反，鯛魚都能維持血壓不變。巯甲丙脯酸是一種血管緊張素轉化酶的抑制劑，當

注射入魚的血液裏後，可以降低血壓。 這顯示腎素-血管緊張素系統有著負責維持基底血壓的作用。經過甲丙脯酸的處理後，把鯛魚直接由千分之三十三轉入千分之六又或進行相反鹽度轉變，也不會影響鯛魚的血壓穩定性。 由此可知，血壓的穩定性不只是由腎素-血管緊張素系統控制。在低滲透壓生活的鯛魚的飲水量比在高滲透壓生活的鯛魚爲低，注射甲丙脯酸或把鹽度千分之三十三的鯛魚突然轉移至千分之六的水裏，都可以降低鯛魚的飲水量，這說明了腎素-血管緊張素系統對鯛魚的飲水量有著極大的影響。 注射血管緊張素可以增加在低滲透壓生活的鯛魚的飲水量，但等同的份量卻降低了生活在高滲透壓鯛魚的飲水量，使用血管抗張藥亞硝醯鐵氰化鈉，刺激內源的腎素-血管緊張素系統，能使生活在高滲透壓鯛魚的飲水量降低，但卻使生活在低滲透壓的鯛魚的飲水量增加。本研究討論腎素-血管緊張素系統和激肽釋放酶-激肽系統控制血壓和飲水量的關係，從而嘗試解釋血管緊張素在不同鹽度下的雙樣式作用。

Content

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Abbreviation

Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
RAS	Renin angiotensin system
KKS	Kallikrein kinin system
ACE	Angiotensin converting enzyme
SNP	Sodium nitroprusside
ACTH	Adrenocorticotrophic hormone
GFR	Glomerular filtration rate
HHL	Hippuryl-L-histidyl-L-leucine
MS-222	3-aminobenzoic acid ethyl ester
IsoHb	Iso-hemoglobin
PaO_2	Partial pressure of oxygen of arterial blood
PvO_2	Partial pressure of oxygen of venous blood
P_{50}	Partial pressure of oxygen of blood at 50% saturation
Ppt	Parts per thousand
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophroesis
ECFV	Extracellular fluid volume

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Chapter 1

General Introduction

Fishes are classically classified into stenohaline and euryhaline by their tolerance in different salinities. Euryhaline fish such as eels and salmons can survive in both hyper- and hyposmotic media. The mechanism for them to cope with the rapid changes in the environmental salinities has been subjected to intensive investigation (Marshall, 1995). Fish are immersed in water but to obtain osmotic-free water in hyperosmotic media is energy consuming. Classically, marine fish need to drink seawater to compensate for the osmotic loss and therefore drinking of hyperosmotic-adapted fish are usually higher than that of hyposmotic-adapted fish. Seawater intake is desalinated along the intestine and the ions are excreted by energy-dependent Na^+/K^+-ATPase in the intestinal epithelium and gill chloride cells. This process eliminates the ions in seawater until it is dilute enough for absorption (Loretz, 1995). The movement of water in hyper- and hyposmotic media are opposite: in seawater, water is lost to the external environment but water enters the body in freshwater environment. These water movements will have an effect on the extracellular fluid volume and therefore, blood pressure and blood volume would also vary under rapid salinity changes (Chan *et al.*, 1978). The renin-angiotensin system (RAS) is an endocrine system that is responsible for volume and pressure homeostasis

in vertebrates. Angiotensin is an active pressor substance and a potent dipsogen in fish (Olson, 1992). Some studies have shown that regulation of the blood pressure at rest or exercise are partially related to the RAS (Platzack *et al.*, 1993). The status of this system also leads to the changes in drinking appetite of the animals in general (Kobayashi and Takei, 1996). Many studies have shown that the drinking rate in teleost is tightly regulated by RAS, especially during seawater inhabitation (Malvin *et al.*, 1980; Carrick and Balment, 1983; Balment and Carrick, 1985). However, recent investigations have noted controversy on the relationship between the RAS and the dipsogenic behavior in teleost (Takei, 2000). Angiotensin is not always dipsogenic in fish but may have no effect or even inhibit the drinking rate of fish (Kobayashi *et al.*, 1983). Many classical effects of RAS inhibition by angiotensin-converting enzyme (ACE) inhibitors are found not to be related to a lowering of angiotensin level, but related to an elevated kinin level. For regulation of blood pressure in fish, studies using anti-angiotensin antibodies also demonstrated the circulating angiotensin levels have no direct on the stability of blood pressure in the eel (Takei, and Tsuchinda, 2000).

Among teleosts, physiological studies are mainly focused on a few experimental models such as eel, trout, salmon, flounder, tilapia, etc. The osmoregulation studies of teleosts are based on those well-known euryhaline species that most of them are

actually atypical in teleosts. Typical marine teleost models for experimentation are lacking due to difficulties in captive culturing. Silver seabream (*Sparus sarba*) is found to be a physiological euryhaline species and they are able to survive in captive condition for a long period (Woo and Kelly, 1995). The general physiological and osmoregulatory responses in seabream are found to be comparable to other euryhaline species. The ability for seabream to cope with abrupt salinity challenges is high and even better than those well known euryhaline species such as eels in terms of rapid achievement of ion homeostasis (Kelly and Woo, 1999a). The relationship among blood volume , blood pressure and drinking rate that are important parameters during salinity adaptation in seabream has not been addressed. Moreover, the metabolic cost of osmoregulation varies among salinity adaptation, but studies of hemoglobin characteristics in this field are lacking. Also, whether RAS is involved in the regulation of these parameters and how they relate to the euryhalinity of seabream are open areas for investigation.

In this thesis, focus will be on the effect of salinity on the drinking behavior and cardiovascular responses including oxygen dissociation curve, blood pressure and blood volume, and further demonstration of the role of RAS in the events using seabream as a model. Chapter 2 in the present studies will focus on the effect of different salinities on some the cardiovascular characteristics such as blood volume

and blood pressure. These parameters are susceptible to changes under various osmotic conditions. The drinking rate of seabream will also be investigated to see how the fish responds under different environmental salinities (Kobayashi *et al.*, 1983; Malvin *et al.*, 1980). Oxygen dissociation curves of seabream is also under investigation to study the respiratory characteristics of seabream under different energy demand for osmoregulation in different salinities. In chapter 3, possible role of RAS in the regulation of blood pressure and drinking rate in seabream is investigated. Evaluation of these parameters will be through the manipulation of the RAS by administration of angiotensin, ACE inhibitors and vasodilator for endogenous activation of RAS. The findings in the present studies will be discussed in context with our knowledge towards the RAS in fish and osmoregulation of seabream. A novel perspective of RAS in teleostean physiology will be presented and the significance of another endocrine system, the kallikrein-kinin system (KKS) on seabream euryhalinity will be discussed.

Chapter 2

Effects of salinity on the cardiovascular responses and dipsogenic behaviors of silver seabream, *Sparus sarba*.

2.1 Literature review

2.1.1 Teleost euryhalinity

There is an increasing number of studies on the adaptation and tolerance of marine fish to low salinity environments (Wu and Woo, 1983; Woo and Chung, 1995; Kelly and Woo, 1999a; Kelly and Woo, 1999b; Kelly *et al.*, 1999a). Many marine teleosts are found to be more euryhaline than expected. It was demonstrated that coral reef angelfish can tolerate hyposmotic media (Woo and Chung, 1995) and black seabream can survive from hypersaline to freshwater (Kelly *et al.*, 1999a). These studies supported the view that teleosts are originated from freshwater as they retain the osmoregulatory machinery from their ancestors (Woo and Chung, 1995). Considering the hypothesis that kidney was primarily developed for osmoregulation in freshwater, as nitrogenous waste is usually excreted in the form of ammonia through the gills of teleosts, the freshwater origin of marine teleosts has become crystal clear. Based on these studies, it seems that the concepts of stenohalinity and euryhalinity have to be revised: species that live in a stenohaline environment because of their ecology should be referred as "ecologically stenohaline"; while those having

the ability to live in fluctuating salinities should be referred as "physiologically euryhaline" (Woo and Chung, 1995).

Silver seabream (*Sparus sarba*) has been shown to be a physiologically euryhaline species and can survive from hypersaline (50 ppt) to hyposmotic (6 ppt) media (Kelly and Woo, 1999b) and even freshwater (0 ppt) (Li and Woo, unpublished observation). Hyposmotic adaptation did not significantly alter serum Na^+, Cl^- or muscle moisture content. The chloride cell morphology through SEM studies showed a typical freshwater type under hyposmotic adaptation. The branchial Na^+/K^+-ATPase activity of both silver (Kelly and Woo, 1999b) and black seabream (Kelly *et al.*, 1999b) was found to be higher in seawater than freshwater, a phenomenon which is compatible to the observations in other euryhaline fish such as killifish (Pickford *et al.*, 1970), tilapia (Dange, 1986) and eel (Epstein *et al.*, 1971). The study of abrupt hyposmotic exposure also revealed that the ability of silver seabream to adapt to low salinities is unexpectedly high and fast (Kelly and Woo, 1999a). The chloride cell morphology changes from a seawater sunken type to a freshwater protruding type within 3 hours of hyposmotic adaptation. Serum osmolytes such as glucose, protein, urea also elevated during the acute period of hyposmotic adaptation. These results suggested that silver seabream, although is a true marine fish, is a good model for studying euryhalinity in teleost.

2.1.2 Salinity and blood respiratory properties

It was well documented that fishes commonly exhibit hemoglobin (Hb) multiplicity. Different "isoHb" may occur in the same individual at the same or different stages of its development (Wilkins and Iles, 1966). Hb multiplicity may result from either gene-related heterogeneity or nongenetic heterogeneity. This is in contrast to mammalian Hb in which there is only a single main Hb component (Kitchen, 1974).

Based on Hb multiplicity, teleostean fish may be differentiated into 3 classes. Class I fish possess electrophoretically anodal Hbs that have normal Bohr, Root, phosphate, and temperature effects (e.g. Carp; Weber and Lykkeboe, 1978). Class II fish possess Hbs that consist both anodal component (as in Class I) and cathodal one, which exhibit high oxygen affinities and small, often reverse Bohr effect (high affinity is maintained by low pH) and temperature sensitivities (e.g. trout and eel; Binotti *et al.*, 1971; Weber *et al.*, 1976). Class III fish possess Hbs that appear to be sensitive to pH but insensitive to temperature (e.g. tuna; Rossi-Fanelli *et al.*, 1960). The functions of different types of Hb were reviewed by Jensen *et al.* (1998).

Tun and Houston (1986) and Marinsky *et al.* (1990) investigated the effects of environmental factors on iso-hemoglobin (isoHb) abundance within individual fish and found increases in individual components in trout subjected to variable conditions

of temperature, oxygen availability, and photoperiod. However, no study has been performed to investigate the effects of salinity on isoHb abundance. As the cost of osmoregulation in different salinities are different, those that are living in extreme salinities (freshwater and hypersaline) invest more energy for osmoregulation than those in isosmotic medium. One would expect that changes might occur in Hb components to cope with the different oxygen consumption in different salinities. In fact, it was already shown that the total hemoglobin concentration in extreme salinities is higher than the isosmotic medium in seabream (Li and Woo, unpublished data). Further investigation is needed to find out whether isoHb also change when the fish inhabits different salinities.

2.1.3 Salinity and blood volume

Estimation of blood volume is usually carried out by 2 major methods. As the blood is composed of 2 compartments, the blood cells and plasma, determination of the volume of either blood cells or plasma can result in estimation of blood volume through concomitant haematocrit measurement. There were attempts to measure the plasma volume by using indicator dilution method. Extracellular space markers such as Evans blue dye (T_{1824}), ^{125}I-albumins, inulin, mannitol, and sucrose have been frequently used for estimation of plasma compartment. Blood cell volume is usually measured by radiolabeled red cell (Conte *et al.*, 1963). This method requires

repeated blood sampling from a single fish, and the volume of the specific compartment is estimated by extrapolating the concentration of the indicator to time "zero" to find the instantaneous mixing concentration. Alternative methods were also used in blood volume studies. Estimation of blood volume by single exponential function allowed determination of blood volume of the same individual at different time intervals (Takei, 1988; for review, see Olson, 1992).

A number of studies have been done to estimate the resting blood volume in different osteichthyean species, and a summary is given in Table 2.1 (p.16). From Table 2.1, the blood volume of teleosts usually ranges from 2-4% body weight, with some exception such as tuna, *Thunnus alaunga* which has a blood volume over 13% body weight. Such exception might be due to its extensive heat-exchanging rate (Laurs *et al.*, 1978) and high metabolism. Generally, the blood volume of osteichthyes is the lowest among vertebrates. Few studies have been carried out to investigate the relationship between salinity and blood volume of fish though blood volume is the extracelluar fluid volume (ECFV) parameter that is most susceptible to salinity challenge. Nishimura *et al.* (1976) found that the blood volume and haematocrit of eel are not affected when the eels are transferred from seawater to freshwater for 1,2 and 5 weeks. The blood volume appears to be well maintained in both hydrating and dehydrating environments.

2.1.4 Salinity and blood pressure

Few studies have addressed the relationship between blood pressure and salinity adaptation in fish. A summary of osteichthyean blood pressure is given in Table 2.2 (p.17). From Table 2.2, the blood pressure of some common experimental fish models ranges from 20-40 mmHg, which is the lowest among the vertebrates. Such phenomenon might be related to the low blood volume observed in fish. Moreover, most of the studies concerning blood pressure in teleost focus mainly on a few experimental models such as eels and trouts, so the understanding of the interspecific relationship of blood pressure in fish is lacking. A higher resting blood pressure in freshwater eel than in seawater eel was observed but the physiological significance has not been discussed (Tierney *et al.*, 1995). Chester Jones *et al.* (1969) also found that the freshwater resting dorsal aortic pressure for silver eel is higher than the seawater one. When the eel was transferred from freshwater to seawater, the blood pressure dropped to seawater level within 2 hours. In Japanese eel, abrupt transfer from freshwater to seawater caused a drastic drop in blood pressure over 30 %. During the acute acclimation period, blood pO_2 decreased while blood pCO_2 and ventilation rate increased, indicating salinity challenge has direct effect on the cardiovascular and respiratory responses (Chan *et al.*, 1978). Though the significance of salinity and RAS (see chapter 3) has been noted by several

investigators (Olson, 1992; Kobayashi and Takei, 1996), the direct effects of salinity on blood pressure is still an opened area for investigation.

2.1.5 Intestine physiology

Seawater fishes lose water to the external environment and compensate for the loss by drinking. The drinking rate in seawater fish is usually higher than the freshwater fish, but the absolute amount is dependent on species (Kobayashi *et al.*, 1983). A summary of drinking rate in osteichthyes is given in Table 2.3 (p.18). When fish drink seawater, the fish need to get rid of the salt before they can obtain osmotic-free water. Desalinization is the process that removes the salt from the ingested water to an isosmotic concentration. Desalinization occurs through both passive and active transport of NaCl from the lumen. In the esophagus, the water permeability is less than that of the intestine, limiting the energetically expensive recycling of water. In eel, for example, the freshwater-adapted esophagus is impermeable to water, Na^+ and Cl^-, while upon seawater adaptation, the esophagus is selectively permeable to Na^+ and Cl^-, but not to water (Hirano and Mayer-Gostan, 1976). In some species examined, the process of desalinization is complete by the time the ingested fluid is delivered to the anterior intestine (Parmalee and Renfro, 1983). Once desalinization is complete, the anterior intestine is responsible for the active transport of NaCl. This establishes a water potential gradient that drives water

uptake from the lumen.

Ion transport in the intestine has been mainly studied through the short circuit current electrophysiological technique (for review, see Loretz, 1995). Active transport of salt in the intestine ultimately depends on the serosal membrane Na^+/K^+-ATPase, as ouabain administration abolished ion transport in eel intestine (Marvao *et al.*, 1994; Trischitta *et al.*, 1992). Studies using immunostaining of Na^+/K^+-ATPase also demonstrated that this enzyme is mainly located at the serosal membrane (Seidelin *et al.*, 1999). Na^+ is driven inward into the cell by the Na^+/K^+-ATPase, creating a large electrochemical gradient across the membrane. Na^+, K^+, and Cl^- are transported into the cell across the mucosal membrane by non-energetic $Na^+/K^+/2Cl^-$-cotransporter (Aguenaou, *et al.*, 1989; Trischitta *et al.*, 1992). Treatments of furosemide or bumetanide, which are the $Na^+/K^+/2Cl^-$-cotransporter inhibitors, prevent the ion transport of the intestine only at the mucosal membrane (Marvao *et al.*, 1994). The K^+ and Cl^- that accumulated inside the cell might be transported inward through K^+ channel, Cl^- channel, Cl^-/HCO_3^- exchange (Zuidema *et al.*, 1985). The gain in salt through this active process is excreted by the chloride cell in the gills to the external environment again. Considering the mechanism of water uptake in the intestine, the only energetically active component is the Na^+/K^+-ATPase (Loretz, 1995), this implies that the water

uptake ability should be strongly related to the activity of this enzyme. In fact, the intestinal Na^+/K^+-ATPase activity in seawater fish is higher than that in freshwater fish, indicating the functional importance of intestine in seawater survival (Kelly *et al.*, 1999a).

Ando and Nagashima (1996) have investigated the stimulus of drinking in eel using an esophagus-cannulated preparation. It was shown that Na^+-containing perfusates stimulate drinking while Cl^--containing perfusates inhibit drinking. The authors suggested that a self-regulating mechanism is present in the eel intestine to regulate drinking. When seawater entered the intestine, high Cl^- level inhibits drinking to allow the fluid to stay longer in the intestine for desalinization. When the salt is lowered to a threshold level, Na^+-induced drinking may accelerate water intake again. This is supported by the observation that when the eel was placed in hypertonic sucrose or mannitol solution, drinking rate dropped to a very low level (Hirano, 1974). This observation indicated that ions might be a stimulus to induced drinking.

The hormonal modulation of ion and water transport in the intestine also received some attention, although the number of studies is still very limited. The permeability of intestine towards water is usually higher in seawater than in freshwater fish. Prolactin, a freshwater-adapting hormone (Pickford and Phillip,

1959), was found to lower the water uptake ability of the seawater-adapted intestine to a freshwater level (Morley *et al.*, 1981). Interestingly, prolactin treatment in seabream led to general elevations of the intestinal Na^+/K^+-ATPase activity, in both hyper- and hyposmotic media (Kelly *et al.*, 1999c). This is in contrast to the role of prolactin in freshwater-adaptation as a higher Na^+/K^+-ATPase activity is observed in seawater intestine. However, combining the evidence that the drinking rate in hyposmotic fish is less than that of hyperosmotic one, the authors suggested that such increase in Na^+/K^+-ATPase activity might facilitate ionic absorption from ingested food instead of desalinization of ingested water. In the experiments involving replacement therapy of hypophysectomized killifish, cortisol was shown to restore the Na^+/K^+-ATPase activity in the intestine (Pickford *et al.*, 1970). However, Seidelin *et al.* (1999) found that cortisol treatment in brown trout has no effect on the Na^+/K^+-ATPase activity in both pyloric caeca and posterior intestine, but increases the fluid transport capacity of the posterior intestine. It was suggested that cortisol might induce other biochemical or anatomical changes that stimulate the isosmotic fluid transport capacity in the trout intestine.

1.1.6 Summary

A number of studies have shown that marine fish are capable of surviving in hyposmotic media and the concept of euryhalinity has recently received challenge.

Silver seabream, by definition, is a "physiologically euryhaline" species and there is increasing evidence for establishing this species as a good model for studying osmoregulation. The blood volume and blood pressure are highly regulated in fish and they are important parameters to consider in the face of osmotic disturbance. Few studies have demonstrated the direct effects of salinity on the blood volume and blood pressure. The drinking rate of seawater fish is usually higher than the freshwater fish as the former needs to compensate the osmotic water loss in a hyperosmotic media. The mechanism of water intake by the intestine requires desalinization in esophagus and stomach and active transport of ions in the anterior intestine. Overall ion uptake in the intestine is driven by the Na^+/K^+-ATPase located at the serosal membrane but the details of ion movement are still under investigation. The appetite for drinking is controlled by the level of ion concentration in the intestine suggesting the presence of a self-regulating mechanism in fish. Studies of hormonal regulation of intestine physiology are few but cortisol has been found to increase the fluid transport capacity while prolactin lower it by altering the permeability of the intestinal membrane.

Table 2.1. Blood volume of different osteichthyes. (Modified from Olson, 1992)

Species	Environment	Blood volume (ml/kg)	Reference
Acipenser fluvescens	FW	37	Thorson (1961)
Polyodon spathula	FW	30	Thorson (1961)
Lepioteus patostomum	FW	38	Thorson (1961)
Amia calva	FW	34	Thorson (1961)
Catastomus commersoni	FW	22	Thorson (1961)
Ictiobus cyprinellus	FW	28	Thorson (1961)
Pseudoscarus guacamaia	SW	36	Thorson (1961)
Epinephelus striatus	SW	26	Thorson (1961)
Gymnothorax funebris	SW	22	Thorson (1961)
Lutianus campechanus	SW	22	Thorson (1961)
Lutianus griseus	SW	20	Thorson (1961)
Mycteroperca tigris	SW	33	Thorson (1961)
Sphyraena barracuda	SW	28	Thorson (1961)
Atractosteus trisechus	FW	31	Siret *et al.* (1976)
Clarias batrachus	FW	47	Pandey *et al.* (1978)
Neoceratodus forsteri	FW	49	Sawyer *et al.* (1976)
Anguilla japonica	FW	35	Takei (1988)
Anguilla rostrata	SW	28	Nishimura *et al.* (1976)
	FW	29	Nishimura *et al.* (1976)
Cyprinus carpio	FW	30; 53	Thorson (1961); Avtalion *et al.* (1974)
Heteropneustes fossilis	FW	13-20	Pandey *et al.* (1976)
Oncorhynchus nerka	FW	54	Smith (1966)
Oncorhynchus mykiss	FW	28-35; 41	Conte *et al.* (1963); Nichols (1987)
Oncorhynchus mykiss	SW	69	Smith (1966)
Oncorhynchus kisutch	SW	62	Smith (1966)
Salvelinus fontinalis	FW	45	Nichols *et al.* (1985)
	SW	44	Nichols *et al.* (1985)
Salvelinus namaycush	FW	43	Hoffert (1966)
Amphipnous cuchia	FW	31	Munshi *et al.* (1975)
Channa punctatus	FW	30	Sinha and Munshi (1981)
Enophrys bison	SW	71	Sleet and Weber (1983)
Seriola quinquerdiata	SW	47	Yamamoto *et al.* (1980)
Pomatomus saltatrix	SW	43	Ogilvy *et al.* (1988)
Thunnus alalunga	SW	132	Laurs *et al.* (1978)

FW = Freshwater ; SW = Seawater

Table 2.2. Blood pressure of different osteichthyes.

Species	Environment	Mean blood pressure (mmHg)	Reference
Salmo gairdneri	FW	33 (da)	Gray and Brown (1985)
Amia calva	FW	25 (da)	Butler *et al.* (1995)
Anguilla rostrata	FW	23 (da)	Butler and Oudit (1995); Bernier *et al.* (1999); Oudit and Butler (1995)
	FW	30 (da)	Nishimura *et al.* (1978)
Oncorhynchus mykiss	FW	27 (da)	Bernier *et al.* (1999a);
		20-27 (da)	Bernier *et al.* (1999b);
		30 (da)	Bernier and Perry (1999)
Anguilla anguilla	FW	23 (da)	Henderson *et al.* (1976);
		33 (va)	Chester Jones *et al.* (1969)
		24 (da)	
	SW	30 (va)	Chester Jones *et al.* (1969)
		18 (da)	
Channa maculata	FW	22 (va)	Yu and Woo (1987)
		18 (da)	
Opanus tau	SW	20 (da)	Nishimura *et al.* (1979)
Pagothenia borchgrevinki	SW	28 (da)	Axelsson *et al.* (1994)
Gadus morhua	SW	29 (va)	Platzack *et al.* (1993)
		22 (da)	
Platichthys flesus	SW	39 (da)	Balment and Carrick (1985)
Anguilla japonica	FW	44 (va)	Chan *et al.* (1978)
		29 (da)	
	SW (3h)	36 (va)	Chan *et al.* (1978)
		19 (da)	

FW = Freshwater ; SW = Seawater ; da = dorsal aorta ; va = ventral aorta

Table 2.3. Drinking rate of different osteichthyes.

Species	Environment	Drinking rate (ml/kg/h)	Reference
Pseudorascora parva	FW	2.78	Kobayashi *et al.* (1983)
Rhodeus ocellatus	FW	3,76-6,38	Kobayashi *et al.* (1983)
Cobitis anguillicaudatus	FW	2.11	Kobayashi *et al.* (1983)
Lampetra japonica japonica	FW	0	Kobayashi *et al.* (1983)
Salvelinus leucomaenis	FW	0.2	Kobayashi *et al.* (1983)
Cyprinus carpio	FW	0.12	Kobayashi *et al.* (1983)
Ctenopharngodon idella	FW	1.9	Kobayashi *et al.* (1983)
Sarotherodon mossambicus	FW	0.93	Kobayashi *et al.* (1983)
Rhodeus lanceolatus	FW	1.54	Kobayashi *et al.* (1983)
Pungitius sinensis	FW	1.58	Kobayashi *et al.* (1983)
Carassius auratus	FW	3.57	Kobayashi *et al.* (1983)
Oryzia latipes	FW	3.02	Kobayashi *et al.* (1983)
Gambusia affinis	FW	2.33	Kobayashi *et al.* (1983)
Gyrinocheilus anymonieri	FW	3.01	Kobayashi *et al.* (1983)
Leuciscus hakonesis	FW	0.41-0.86	Kobayashi *et al.* (1983)
Carassius carassius	FW	0.56	Kobayashi *et al.* (1983)
Parasilurus asotus	FW	0.1-0.3	Kobayashi *et al.* (1983)
Chaenogobius annularis	FW	0.15	Kobayashi *et al.* (1983)
Tridentiger obscurus	FW	1.55	Kobayashi *et al.* (1983)
Sadinops melanosticta	SW	6.99	Kobayashi *et al.* (1983)
Trachurus japonicus	SW	1.36	Kobayashi *et al.* (1983)
Platichthys bicoloratus	SW	0.75	Kobayashi *et al.* (1983)
Acanthopagrus schlegeli	SW	3.64	Kobayashi *et al.* (1983)
Fugu niphobles	SW	2.02-3.41	Kobayashi *et al.* (1983)
Parpristipoma trilineatum	SW	6.13	Kobayashi *et al.* (1983)
Sebastes inermis	SW	2.43	Kobayashi *et al.* (1983)
Rudarius ercodes	SW	2.07	Kobayashi *et al.* (1983)
Glossogobius giuris giuris fasciatopunctatus	SW	0.53	Kobayashi *et al.* (1983)
Chasmichthys dolichognathus gulosus	SW	2.99	Kobayashi *et al.* (1983)
Sillago japonica	SW	12.7	Kobayashi *et al.* (1983)
Mugil cephalus	SW	4.13	Kobayashi *et al.* (1983)
Callionymus richardsoni	SW	4.92	Kobayashi *et al.* (1983)
Hypodytes rubripinnis	SW	7.00	Kobayashi *et al.* (1983)
Fundulus heteroclitus	SW	8.4	Malvin *et al.* (1980)
	FW	0.8	Malvin *et al.* (1980)
Platichthys flesus	SW	24	Balment and Carrick (1985)
	FW	0.67	Balment and Carrick (1985)
Oncorhynchus mykiss	FW	0.9	Fuentes and Eddy (1998)
Salmo salar	SW	3.8	Fuentes and Eddy (1997)
	FW	0.1	Fuentes and Eddy (1997)
Anguilla anguilla	SW	0.5	Tierney *et al.* (1995)
	FW	0.06	Tierney *et al.* (1995)
Tilapia mossambica	SW	2.7	Potts *et al.* (1967)
	FW	0.6	Potts *et al.* (1967)
Anguilla japonica	FW	0	Oide & Utide (1968); Kobayashi *et al.* (1983)
	SW	0.8	Oide & Utide (1968)

FW = Freshwater ; SW = Seawater.

2.2. Materials and methods

2.2.1 Experimental animals

Silver seabream (*Sparus sarba*) were obtained from local sea cage. Fish were kept in close circulating system in the Marine Science Laboratory, the Chinese University of Hong Kong. All fish received natural photoperiod throughout the experiment. Both sexes were used and their weight ranged from 20 to 400 g. Fish less than 150 g were used in drinking rate experiment whereas fish ranged from 150 to 250 g were used in measurement of blood volume. Fish weights used in blood pressure experiment ranged from 70 to 300 g.

2.2.2 Salinity adaptation

All the fish were adapted to specified salinities (6, 12, 33 and 50 ppt) for at least 4 weeks before any experiment was done. During the adaptation period, the fish were fed *ad libitum* daily with diet formulated by Woo and Kelly (1995). Fish were fasted for 2 days prior to drinking rate experiment.

2.2.3 Drinking rate measurement

Drinking rate was measured using phenol red method as described by Kobayashi *et al.* (1983). Fish were immersed in media of the specified salinity containing 40 mg/L phenol red for 60 or 90 min. They were then removed and killed by cervical transection. The whole intestine was tied up, excised and removed to a petri dish and 3 ml of saline (0.8 % NaCl) was added. The intestine was cut open and washed

thoroughly to extract the phenol red. An extract of 0.75 ml was then added to a 1.5 ml centrifuge tube containing 0.75 ml petroleum ether. After vortex for 10 sec, the tube was centrifuged for 15 min at 5,000 rpm. A 1 ml syringe was used to extract the aqueous bottom layer and 0.5 ml of it was transferred to a centrifuge tube containing 0.5 ml 5 % trichloroacetic acid. After vortex for 10 sec, the tube was centrifuged for 15 min at 5,000 rpm. The supernatant (0.6 ml) was transferred to a centrifuge tube containing 0.6 ml 1 M NaOH. After vortex for 10 sec, the tube was centrifuged for 5 min at 3,000 rpm. The supernatant was transferred for spectrophotometry at 550 nm. Drinking rate of the fish was calculated from the amount of phenol red accumulated in the intestine within a fixed time interval.

Drinking rate was measured in fish adapted to (1) 6, 12, 33, 50 ppt only, (2) abrupt transfer from 33 to 6 ppt for 2 hours prior to drinking rate measurement, (3) abrupt transfer from 6 to 33 ppt for 2 hours prior to drinking rate measurement, and (4) abrupt transfer from 33 ppt to 1 M sucrose solution.

2.2.4 Respiratory characteristics

The oxygen dissociation curves of blood from 6, 12 and 33 ppt fish were constructed by the mixing technique (Edwards and Martin, 1966). Blood samples were obtained from severing caudal vein into a heparinized syringe and were transferred to test tubes containing phosphate buffer saline at pH 7.8. The blood to

buffer ratio was kept constant at 10 : 1 and the blood samples were separated into 2 test tubes. Water saturated air was blown into one portion and humidified N_2 into the other until an equilibrium was reached. The two portions of blood were then mixed in progressively decreasing proportion of oxygenated blood : deoxygenated blood (i.e., 9 : 1, 7 : 3, 5 : 5, and so forth). The PO_2 of the mixed blood samples was then determined by a Blood Gas Analyzer (CIBA-Corning M248) at 25 °C. Blood samples were collected at the efferent branchial artery to determine the arterial blood oxygen tension (PaO_2). Blood samples were also collected at the afferent branchial artery to determine the venous blood oxygen tension (PvO_2).

Blood samples were taken from 6, 12, and 33 ppt-adapted fish from caudal vein into heparinized syringe (3% sodium heparin in physiological saline). 50 μl of blood was diluted into 1 ml physiological saline. The blood cells were washed 3 times with physiological saline and then lysed in 1 ml distilled water. The proteins were denatured by heating at 70 °C for 10 min. The hemolyzates were stored at –20 °C for protein analysis. The hemoglobin were separated by 12 % SDS-PAGE (Sodium dodecyl sulphate - polyacrylamide gel electrophoresis) and stained by Coomasie blue. Cross-linked bovine hemoglobin (Sigma, St. Louis) was used as standard.

2.2.5 Blood volume measurement

Fish (150 - 250 g) were anaesthetized by 3-aminobenzoic acid ethyl ester

(MS-222, Sigma) until breathing movement stopped. The fish was then transferred to operation table and the gill was flushed with dilute MS-222 solution (10 mg/L seawater). Cannulation of afferent branchial artery was modified from Ames *et al.* (1966). Gill was exposed using a retractor and the whole gill arch was tied off distally using silk suture. A sharpened PE-50 polyethylene cannula (Clay-Adams, USA) filled with 1% heparinized physiological saline [composition modified from Rankin and Maetz (1971)] was directly inserted into the afferent branchial artery. The cannula was further secured using silk suture and cyanoacrylate glue. The fish was then allowed to recover in a dark chamber containing aerated water for 24 h before any experiment was done.

Blood volume of the fish was estimated using indicator-dilution method according to Conte *et al.* (1964). Evans blue, T-1824 (2 mg/ml in physiological saline) was injected through the cannula and flushed with heparinized saline until all the blue dye disappeared. Serial blood samples were collected from the same fish at 5, 10, 15, 25 and 35 min through the cannula into heparinized micro-haematocrit tubes and centrifuged immediately. Haematocrit values were determined and the dye concentration in the plasma was determined spectrophotometrically at 605 nm. A graphical extrapolation of plasma dye concentration at time zero was used to calculate the plasma volume. Volume disturbance was minimized by infusion of same amount

of saline after each blood sampling.

Blood volume was measured in fish adapted to (1) 33 ppt only, (2) 6 ppt only, (3) abrupt salinity transfer from 33 to 6 ppt for 3 hours and (4) abrupt salinity transfer from 6 to 33 ppt for 3 hours.

2.2.6 Blood pressure experiment

Fish (70-300 g) were cannulated as described before for blood volume experiment. The cannula was connected to a pressure transducer coupled to a Harvard Biograph System. A three-way stopcock was connected between the transducer and the cannula to flush the cannula with 1% heparinized saline.

The time course of changes in blood pressure were measured in fish with (1) abrupt salinity transfer from 33 to 6 ppt, (2) abrupt salinity transfer 6 to 33 ppt. "Shame" salinity transfers were also carried out by changing media from (1) 33 to 33 ppt and from (2) 6 to 6 ppt.

2.2.7 Statistical analysis

All data are expressed as mean values ± SEM. The data for the different salinity groups were subjected to one-way ANOVA to test for significance followed by a Tukey comparison test to delineate significance among groups. Independent comparisons between treatment and control were subjected to Student's t test with $P<0.05$ to delineate significance between the two groups.

2.3 Results

2.3.1 Drinking rate

A validation experiment was carried out to validate the phenol red method on drinking rate measurement. Fish without immersion in phenol red solution were sacrificed and complete procedures were done to measure the absorbance of "blank" fish. The absorbance of "blank fish" was 0.0026 (n=8), indicating that background absorbance was negligible. The basal drinking rate of silver seabream adapted to different salinities is shown in Fig. 2.1 (p.26). The drinking rate among 50, 33 and 12 ppt fish showed no significant difference while the drinking rate of 6 ppt fish was only half of the other groups. Though one would expect that the fish should drink more in hypersaline environment (50 ppt), but in this case, silver seabream did not drink more in 50 ppt than in 33 ppt. This was consistent with the results obtained using ^{51}Cr-EDTA as indicator for drinking rate measurement (Chow and Woo, unpublished data). The drinking rate measured by isotopes method of seabream was about 1 ml/kg/h in 50, 33 and 12 ppt fish and 0.5 ml/kg/h in 6 ppt fish. When the 33 ppt fish were transferred to 6 ppt for 3 hours, the drinking rate fell by more than 100% [Fig. 2.2 (p.26)]. On the other hand, when 6 ppt fish were abruptly transferred to 33 ppt for 3 hours, the drinking rate increased significantly [Fig. 2.3 (p.26)], but only to a level between the 2 groups that had been adapted to 6 and 33 ppt. When 33 ppt fish

were transferred to 1 M sucrose solution (same osmotic pressure with seawater, but without ions), all fish died within an hour and the phenol red accumulated in the intestine was negligible. This showed that osmotic depletion was quick and lethal to seabream. Also, ions played an important role in regulating drinking in fish.

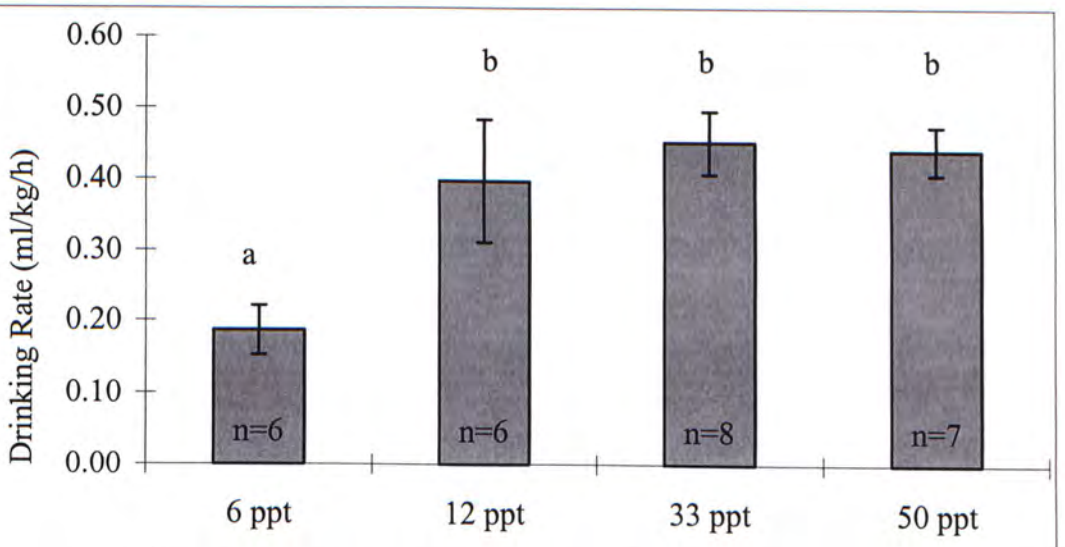

Fig. 2.1. Drinking rate of silver seabream adapted to different salinities. *Denote significant difference (P<0.05) among groups.

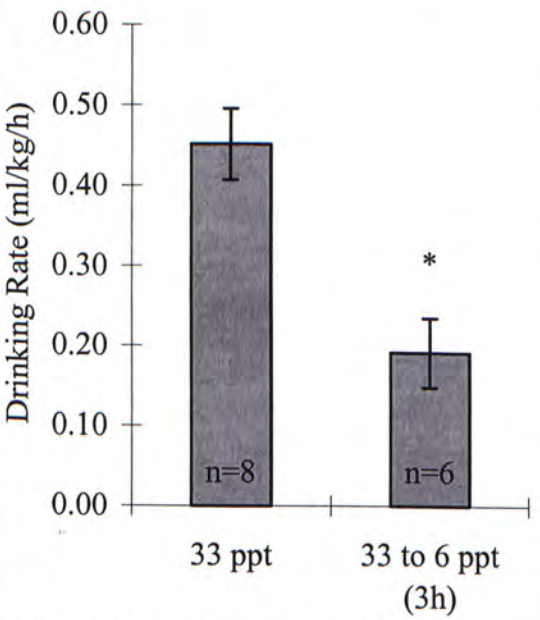

Fig. 2.2. Drinking rate of 33 ppt adapted silver seabream subjected to abrupt hyposmotic transfer. *Denote significant difference (P<0.05) with the control group (33 ppt).

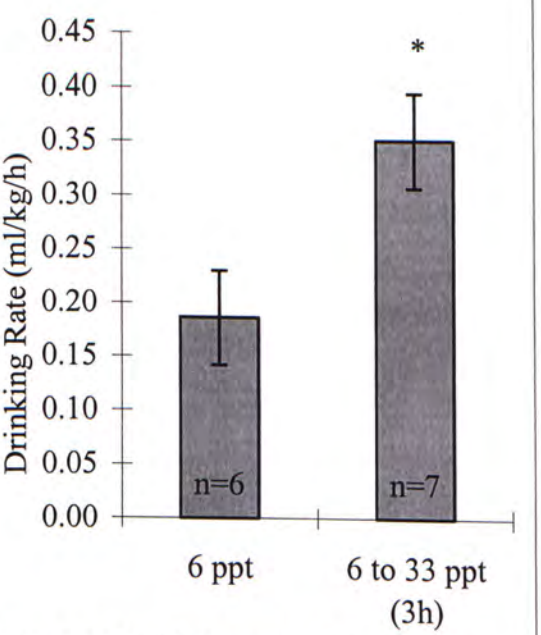

Fig. 2.3. Drinking rate of 6 ppt adapted silver seabream subjected to abrupt hyposmotic transfer. *Denote significant difference (P<0.05) with the control group (6 ppt).

2.3.3 Oxygen dissociation curves

The oxygen dissociation curves of seabream adapted to different salinities showed high levels of similarity [Fig 2.4 (p.28)]. The P_{50} values of all groups were around 13 mmHg. The slope of the sigmoid curve was steep, showing that loading and unloading of oxygen was efficient. The PaO_2 and PvO_2 of seabream adapted to hyposmotic (6ppt) and hyperosmotic (33 ppt) showed no significant difference [Table 2.4 (p.28)]. By fitting the 2 parameters into the oxygen dissociation curve, the oxygenated blood of resting seabream was at about 75 % saturation, while the deoxygenated blood was at 40 % saturation.

The hemolyzate prepared from fish adapted to different salinities showed the same pattern under SDS-PAGE [Fig.2.5 (p.28)]. The banding pattern were similar among groups, which indicated that same types of hemoglobin have been produced by fish adapted to different salinities. The banding pattern of seabream was different from that of bovine standard, showing more than one component or isoHb in seabream blood cell. This pattern of hemoglobin multiplicity is commonly observed in fishes, which favors them to adapt to various habitats that have changing oxygen supply (Jensen *et al.*, 1998).

Table 2.4. Partial pressure of oxygen in arterial and venous blood of 6 ppt and 33 ppt silver seabream.

	PaO_2 (mmHg)	PvO_2 (mmHg)
6 ppt	28.4 ± 2.1	10.4 ± 0.7
33 ppt	24.8 ± 1.1	8.3 ± 0.4

PaO_2 = partial pressure of oxygen of arterial blood ; PvO_2 = partial pressure of oxygen of venous blood.

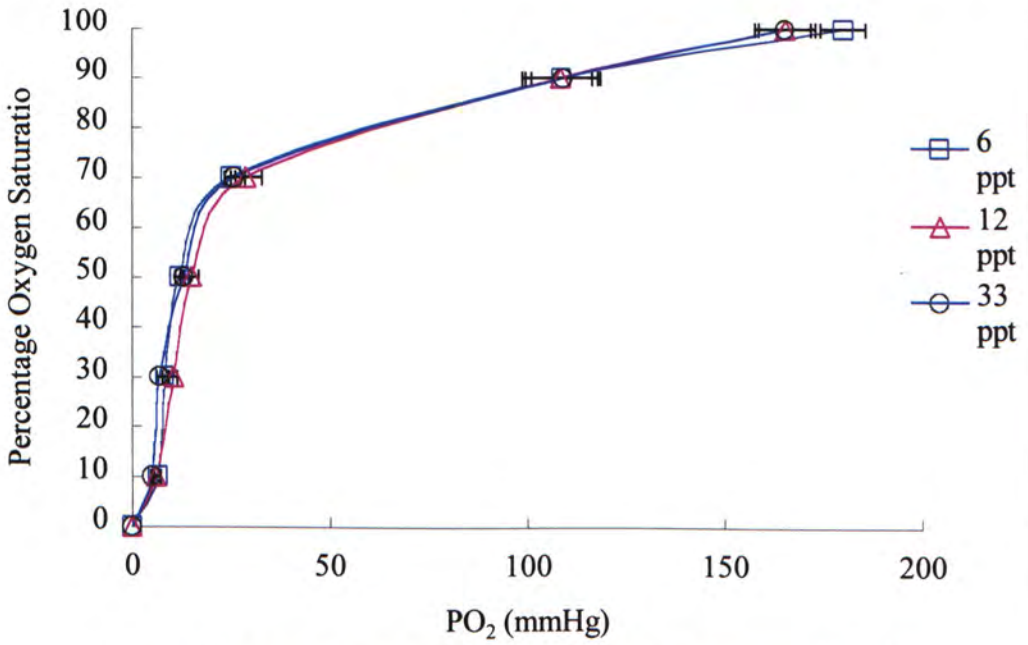

Fig. 2.4. Oxygen dissociation curves of silver seabream adapted to different salinities. (n=6 in each group)

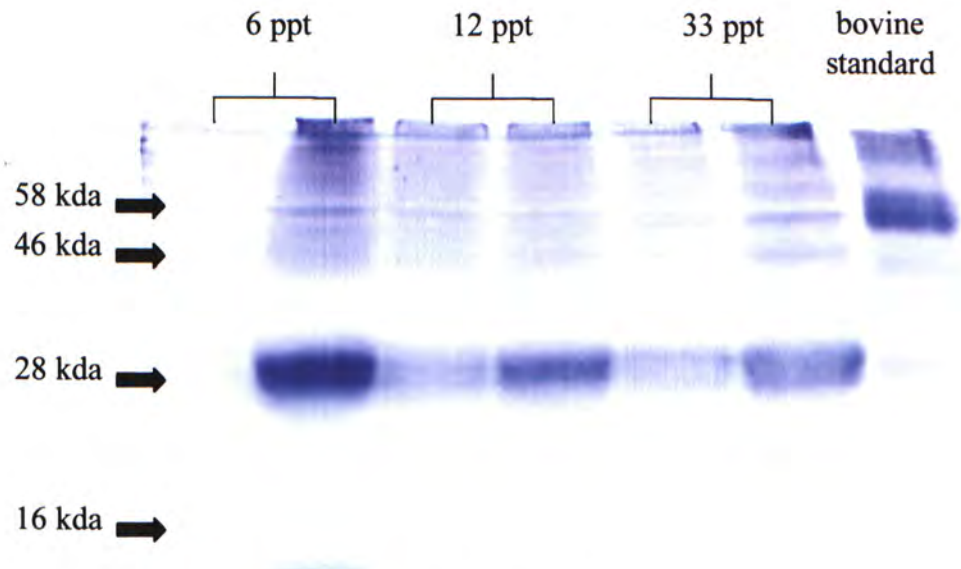

Fig. 2.5. SDS-PAGE (12%) of blood hemolyzates of silver seabream adapted to different salinities.

2.3.3 Blood volume

The blood volume of seabream was around 20 ml/kg body weight in both hyperosmotic (33 ppt) and hyposmotic (6 ppt) media [Fig. 2.7 (p.30); Fig 2.8 (p.30)]. Such blood volume was comparable to those common experimental fish such as eel and trout. A typical curve of the time course of disappearance of Evans blue (T_{1824}) from plasma was shown [Fig. 2.6 (p.30)]. As with other fish species such as the trout (Conte *et al.*, 1963), the rate of disappearance of the dye was slow and this indicated that the dye was bound tightly to the plasma proteins. When the fish were subjected to abrupt transfer from hyperosmotic to hyposmotic media for 3 hours or *vice versa*, the blood volume exhibited no significant changes.

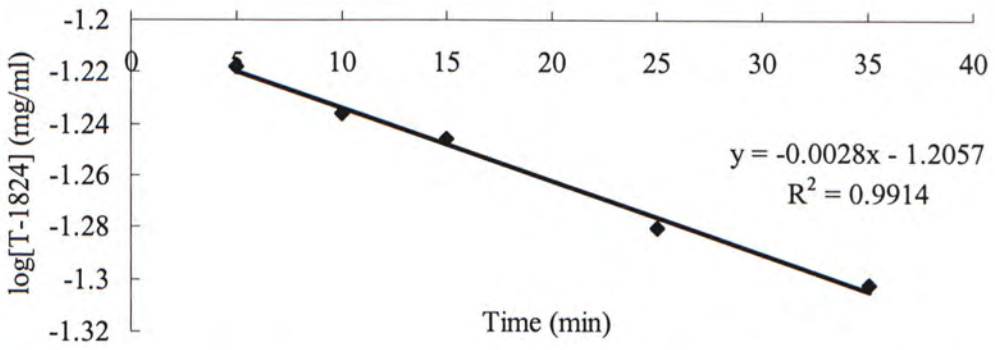

Fig. 2.6. A typical clearance curve of injected T_{1824} with time in seabream. The instaneous mixing concentration of T_{1824} in plasma can be found by extrapolation to zero time.

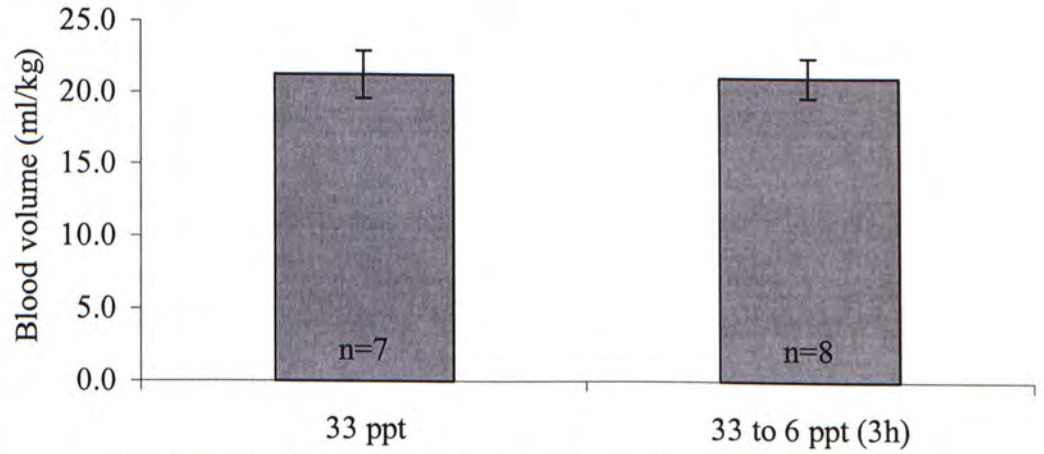

Fig. 2.7. Blood volume of 33 ppt adapted silver seabream 3 hours after abrupt hyposmotic transfer.

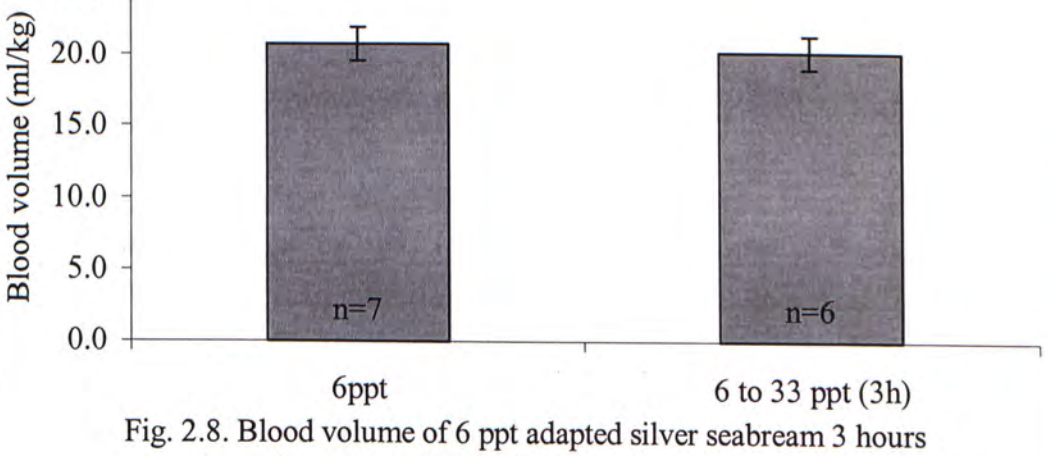

Fig. 2.8. Blood volume of 6 ppt adapted silver seabream 3 hours after abrupt hyperosmotic transfer.

2.3.4 Blood pressure

The mean resting blood pressure of seabream adapted to 33 and 6 ppt showed no significant difference [Fig. 2.9c (p.34) ; Fig. 2.10c (p.35)]. The mean blood pressure ranged from 30 to 40 mmHg and the difference between systolic and diastolic pressure was usually around 10 mmHg. The observed blood pressures were low and compatible to other common experimental models as presented in Table 2.3 (p.18). As the blood pressure of hyperosmotic and hyposmotic seabream has no difference, it is in contrast to that of eel, which has a higher resting blood pressure in freshwater than in seawater (Chester Jones *et al.*, 1969). When the fish was abruptly transferred from 33 to 6 ppt or *vice versa*, the blood pressure showed a slight increase (about 10 mmHg) and then back to resting level within 2 h after the transfer [Fig. 2.9c (p.34); Fig. 2.10c (p.35)]. Comparing the original tracings of the blood pressure during abrupt transfer from 33 to 33 ppt [Fig. 2.9a (p.33)] and from 33 to 6 ppt [Fig. 2.9b (p.33)], the patterns of changes in blood pressure were similar. Similar pattern in original tracings of blood pressure was also observed in the abrupt transfer from 6 to 6 ppt [Fig. 2.10a (p.34)] and from 6 to 33 ppt [Fig. 2.10b (p.35)]. Therefore, the slight increase in blood pressure during and just after the transfer was probably due to the turbulence caused by water current introduced during changing media. The blood pressure continued to maintain at the resting level for 3 h and further (> 24 h) after the

transfer.

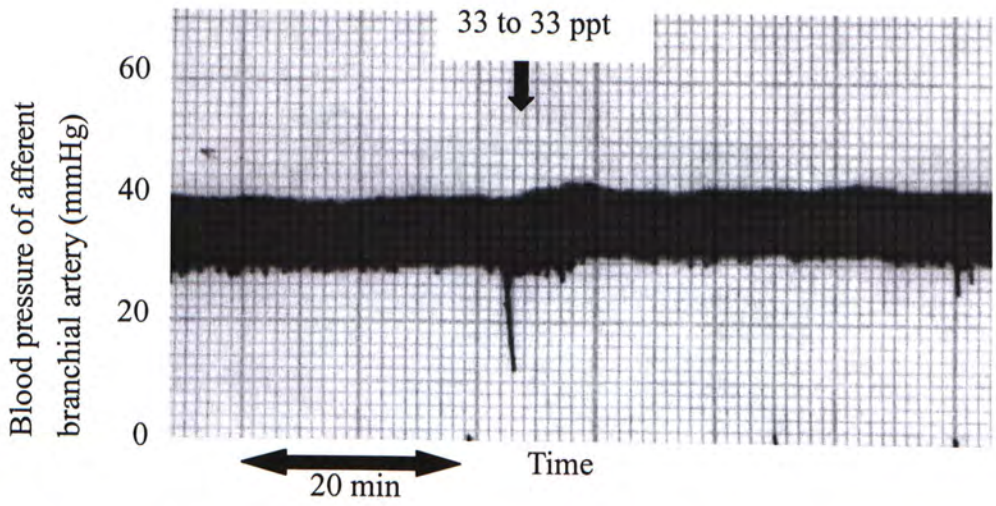

Fig. 2.9a. Original tracing of afferent branchial blood pressure of silver seabream subjected to abrupt transfer from 33 to 33 ppt. (typical example)

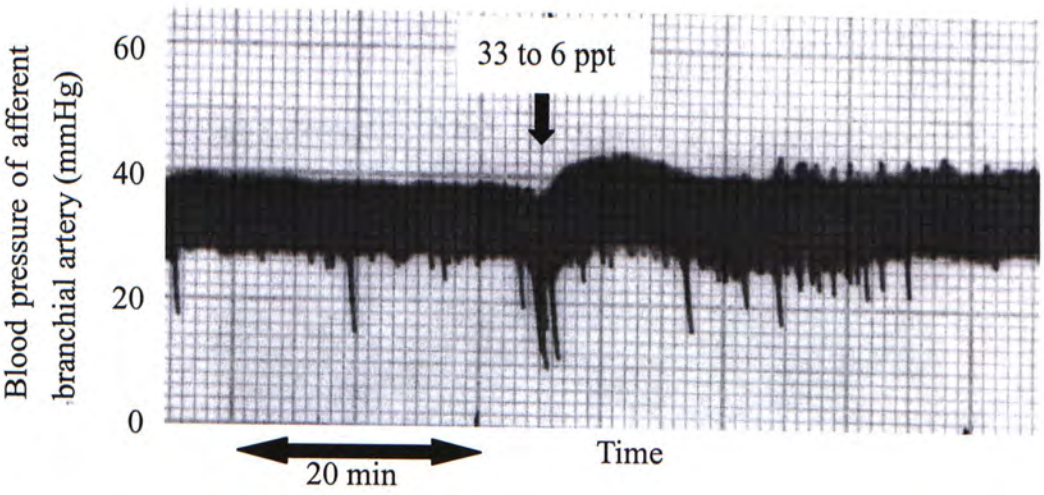

Fig. 2.9b. Original tracing of afferent branchial blood pressure of silver seabream subjected to abrupt transfer from 33 to 6 ppt. (typical example)

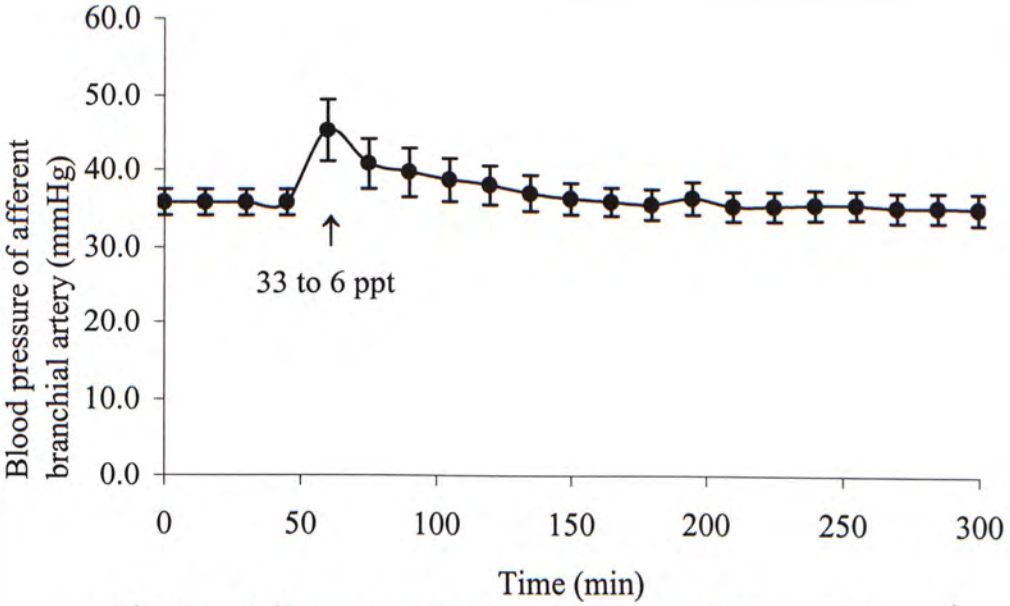

Fig. 2.9c. Afferent branchial blood pressure of silver seabream subjected to abrupt transfer from 33 to 6 ppt. (n = 6)

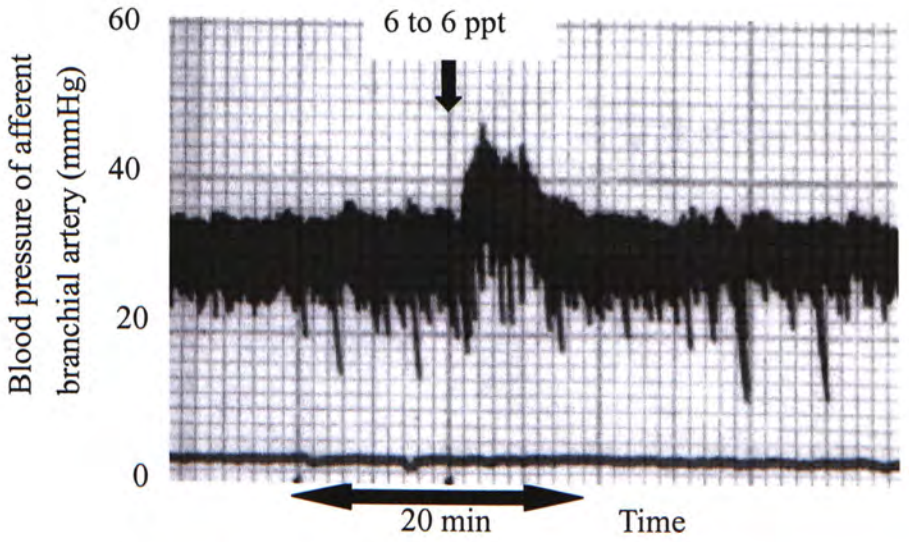

Fig. 2.10a. Original tracing of afferent branchial blood pressure of silver seabream subjected to abrupt transfer from 6 to 6 ppt. (typical example)

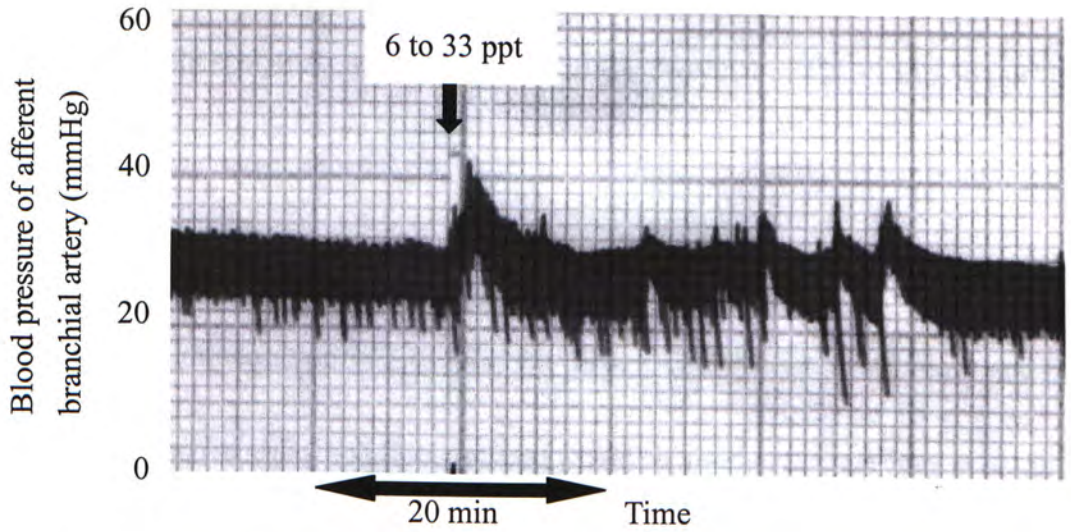

Fig. 2.10b. Original tracing of afferent branchial blood pressure of silver seabream subjected to abrupt transfer from 6 to 33 ppt. (typical example)

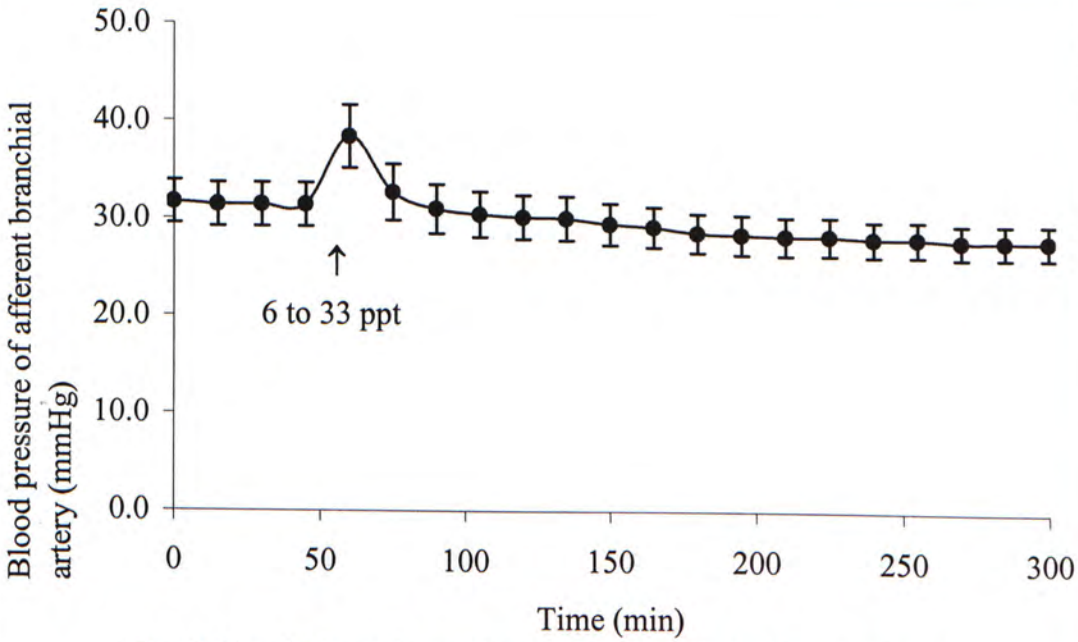

Fig. 2.10c. Afferent branchial blood pressure of silver seabream subjected to abrupt transfer from 6 to 33 ppt. (n=7)

2.4 Discussion

2.4.1 Drinking rate

The drinking rate of 6 ppt seabream was lower than that of 12, 33 and 50 ppt fish. As fish needs to compensate for the water loss in higher salinity, higher drinking rate is expected. However, it seemed that 50 ppt fish should drink more if more water is lost in hypersaline conditions. In fact, the 50 ppt fish did not drink more and this implied that other system may have played a role to conserve water and expel ions. Kelly *et al.* (1999a) found that the branchial and renal Na^+/K^+-ATPase activity in 50 ppt black seabream had no change when compared with 33 ppt fish. However, the visceral somatic index, and the Na^+/K^+-ATPase activity at the anterior gut including esophagus, stomach and pyloric caeca, were higher in 50 ppt fish than that of 33 ppt fish. Elevation of chloride cell number, apical and fractional area in 50 ppt fish was also observed. Such increment in intestinal enzyme activity may facilitate the desalinization of ingested water. The elevated intake of ions is excreted through increased chloride cell function (Kelly *et al.*, 1999a).

When the 33 ppt-adapted fish was abruptly transferred to 6 ppt, drinking rate decreases significantly within 3 hours. On the other hand, when the 6 ppt-adapted fish was abruptly transferred to 33 ppt, drinking rate increase significantly. This indicated that drinking rate is highly regulated in seabream and the drinking control is

rapid in response to salinity changes. The observation that all fish died within an hour in 1 M sucrose solution and the drinking rate was negligible provided further evidence of the importance of drinking and ions are required to stimulate drinking.

2.4.2 Oxygen dissociation curve

The oxygen dissociation curves of fish adapted to different salinities were similar and overlapped together, indicating the existence of same cooperativity and isoHb among them. The results obtained from SDS-PAGE also suggested that same types of hemoglobin have been synthesized under different salinities. A shift in curve usually implies a change in metabolic rate. A left shift in curve may imply decrease in oxygen carrying capacity, but increase in loading and unloading ability, resulting in high tissue oxygen content. This phenomenon is often observed in animals that experience hypoxia, acidosis, nitrite exposure, etc. A right shift is often observed in animals having high metabolic rate and oxygen carrying capacity such as birds and whales (Gratzer and Allison, 1960; Kendrew *et al.*, 1960). The metabolic cost of osmoregulation is higher in extreme salinities (e.g. freshwater and hypersaline) than in isosmotic media (Woo and Kelly, 1995). While it was expected that the oxygen dissociation curve or the isoHb changed to cope with the different metabolic and oxygen demand, the fact is that seabream did not alter the abundance of isoHb but only increased the total hemoglobin content in response to extreme salinities (Li and

Woo, unpublished data).

2.4.3. Blood volume

The blood volume of silver seabream was tightly regulated at 20 ml/kg body weight. Neither long term salinity adaptation nor abrupt osmotic challenge alter the blood volume of this fish. Body hydration as reflected by increase in muscle moisture was observed when seabream was abruptly transferred from 33 to 6 ppt (Kelly and Woo, 1999a). It is expected that blood volume, which is the most susceptible element that is influenced by osmotic disturbance, should change during the acute phase of osmotic adaptation. Such a contention was supported by the results of the blood pressure experiment (see section 2.4.4), in which also no significant changes in blood pressure during abrupt osmotic adaptation were noted. Since the two parameters are highly related (e.g. increase in blood volume usually results in elevation in blood pressure), the present observation suggests that some emergency mechanism for the extrusion of osmotically gained water was present for the regulation of blood volume and pressure. It is hypothesized that the fish sense salinity changes by an indicator other than volume and pressure disturbance by osmotic challenges, as they responded so rapidly that reorganization of osmoregulatory machinery within hours after the abrupt transfer has occurred (Kelly and Woo, 1999a).

2.4.4 Blood pressure

The blood pressure of fish subjected to abrupt osmotic challenge showed no significant change. After the medium has been changed from 33 ppt to 6 ppt or *vice versa*, the blood pressure showed a slight increase and then returned to basal level. This slight increase might be due to the stress induced by the water current during changing media. In contrast to eel, the blood pressure of eel dropped within hours when they were transferred from freshwater to seawater (Chester Jones *et al.*, 1969; Chan *et al.*, 1978). Moreover, during the period of abrupt hyposmotic adaptation period, it had been shown that seabream underwent a series of body reorganization in order to adapt to hyposmotic medium (Kelly and Woo, 1999a). These changes, which happened within 6 hours adaptation, included elevation in serum osmolytes and metabolites such as glucose, protein, urea, and amino acids, increase in chloride cell apical and fractional area, morphological change of chloride cell from a seawater sunken type to a freshwater protruding type, and increase in circulating cortisol level. As a result, the blood pressure of seabream was likely under sophisticated control in different salinities and during adaptation period.

Combining the results of the blood volume and blood pressure experiment, we would like to suggest that these two parameters were under strict control. The sense of salinity changes in seabream should not depend on the changes in blood volume or

blood pressure. As the fish started to reorganize the body structure and function to adapt to different salinities before any changes occurred in blood volume and pressure, it is likely that seabream is more euryhaline than some other well-known euryhaline species such as eel.

Chapter 3

Manipulation of renin-angiotensin system in relation to the cardiovascular responses and dipsogenic behaviors of silver seabream, *Sparus sarba*.

3.1 Literature review

3.1.1 Renin angiotensin system (RAS)

The RAS has been found to be highly conserved throughout vertebrate evolution. A complete RAS has not been found in invertebrates. However, there are studies which have shown that immunocytochemically or chemically active Ang II-like substance was found in annelids. In the nervous system of leeches, *Theromyzon tessulatum* and *Erodella octoculata*, angiotensin-like substances have been identified (Verger-Bocquet *et al.*, 1992; Salzet *et al.*, 1995). The components of RAS in vertebrates are also fairly conserved.

In bony fish, the components of RAS share some homology to mammals. Reviews on RAS in fish by Olson (1992) and Kobayashi and Takei (1996) summarized the basic components as followed. Angiotensinogen, or otherwise known as renin substrate, is a glycoprotein with molecular weight of about 58 KD. It is produced in the liver and secreted into the plasma to act as a precursor for angiotensin. Renin, a highly specific aspartyl proteinase that is released from juxtaglomerular cells in the kidney, hydrolyzes angiotensinogen to Ang I. Ang I is a

decapeptide that is considered as biologically inactive. Ang I is quickly converted into Ang II by ACE located mainly in the gill of fish. Ang II, the biologically active form, is an octapeptide that has widespread effects on the cardiovascular system, renal function, and drinking behavior, etc. In fish, no report has shown that either aldosterone was present or Ang III played a physiological role in conserving Na^+. A summary of components of RAS is given in Fig. 3.1 (p.43).

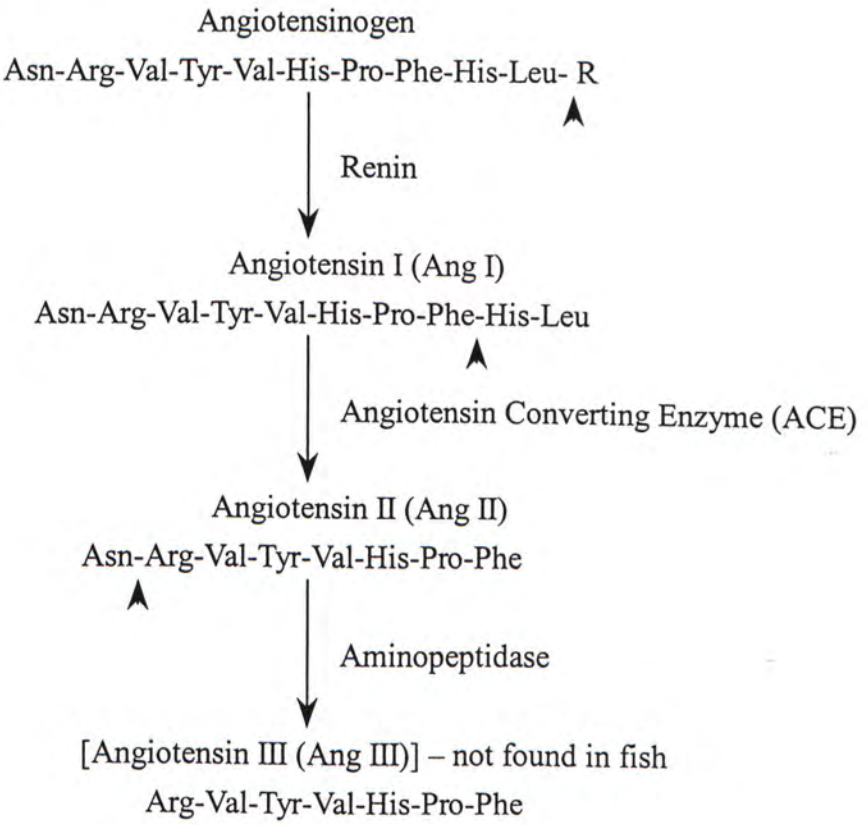

Fig. 3.1. General components of renin-angiotensin system in teleost. The amino acid sequence is based on teleostean data. Short arrowheads indicate the cleaving site of the peptide by the enzyme. (Modified from Kobayashi and Takei, 1996)

Olson (1992) summarized and compared the sequence of angiotensin II in fish and mammals and found that there are only 2 amino acid differences between fish (Asn^1, Val^5) and mammalian (Asp^1, Ile^5) Ang II. Considering the similarity of the amino acid sequence of angiotensin across the vertebrates, this peptide was subjected to great evolutionary constraint as only a few variations were found among them. Only a little mutation in the gene for Ang II production may lead to the deterioration of the individual and the gene pool. Nishimura (2001) also summarized the relationship between the structure and function of angiotensin and angiotensin receptors. The author stated that the variations in amino acid sequence at position 1 and 5 in angiotensin only led to relatively minor effects with respect to function and binding. Moreover, Takei *et al.* (1998) found that tetrapod-type angiotensin ([Asp^1] angiotensin) is present in bowfin, while the Ang II receptors have not yet fully coevolved with the homologous [Asp^1] peptide. The Ang II formed from [Asp^1] angiotensin found in bowfin is completely identical to those found in ox, fowl, snake, turtle, alligator, bullfrog but different from that of the rat (Kobayashi and Takei, 1996). While a tetrapod-type angiotensin is found in the bowfin, a teleost-type angiotensin is found in Australian lungfish (Joss *et al.*, 1999). Although the RAS in vertebrates is structurally conserved, antibodies to human, rat, and mouse renins do not react with salmon tissues (Christensen *et al.*, 1989a), showing species-specific

immuno-reactivity of RAS components.

ACE was found to be located mainly in the gills of bony fish. In the isolated perfused gill system of trout, over 60 % of Ang I was converted into angiotensin II in a single passage through the gills (Olson *et al.*, 1986). Studies using autoradiography showed that ACE was found at the pillar cells of trout gill, especially in the medial border of the respiratory lamellae (Olson, *et al.*, 1989). Comparison of the ACE activities of different trout tissues also revealed that the gill is the major site for conversion (Galardy *et al.*, 1984). Compared to mammals, where the ACE activities are mainly situated on the luminal surface of pulmonary endothelial cells (Ng and Vane, 1967; Ryan *et al.*, 1975), it is interesting that the ACE activity is found in the respiratory organs in both groups of animals. Considering the development of gill and lung are different, the localization of ACE in gill and lung must be analogous but not homologous. Olson *et al.* (1989) suggested that the analogy of the localization could be related to the fact that both respiratory organs were located right before entering the systemic circulation, making them perfect places for regulation of circulating angiotensin level. As Ang II is degraded quickly and the half-time for circulating Ang II in trout is only 3-7 min (Kellogg and Olson, 1990), it is necessary that the peptide is generated right before entering the systemic circulation.

Captopril (SQ 14225), an ACE inhibitor, prevents the formation of Ang II from

Ang I and thus can decrease the circulating Ang II level. Many other ACE inhibitors such as enalapril (Fuentes and Eddy, 1997), competitive inhibitors such as SQ 20881 and P113 (Malvin *et al.*, 1980) have also been employed in studies on the RAS.

To measure the levels of circulating renin and angiotensin, only 2 methods have been documented, i.e. the bioassay and RIA methods. Renin activity can be determined indirectly by the rate of angiotensin formation. In the bioassay method, the angiotensin formed by incubation of plasma with angiotensinogen was eluted from a resin column. The amount of angiotensin in the elutate was determined by the pressor activity on nephrectomized, pentolinium-treated rats (Boucher *et al.*, 1967; Henderson *et al.*, 1976). As this kind of procedure is complicated, RIA method is adopted recently to replace the bioassay method. The RIA method uses human Ang I antibody to determine the amount of angiotensin in the plasma or incubation mixture directly (Nishimura *et al.*, 1979).

In vitro incubation of trout kidney slices with Ang II inhibits renin secretion, suggesting the presence of a negative feedback of renin secretion within the kidney (Bailey and Randall, 1981). Ang II not only inhibits the secretion of renin, but also inhibits renin production at the gene level. Administration of Ang II to rat kidney lowered the renin mRNA and total RNA level while enalapril caused an increase in renal renin mRNA abundance (Johns *et al.*, 1990). It was proposed that Ang II

exerted a direct inhibitory effect on renin by regulation of renin gene expression in renal vasculature. However, further investigations are needed to confirm whether this is also true in fish, and the relationship between this negative feedback and status of RAS including basal and activated status.

3.1.2 RAS and blood pressure

It is generally known that RAS is important in pressure and volume homeostasis. Its effects on Na^+ conservation and aldosterone regulation have received considerable attention in mammals. As aldosterone has not been identified in fish, the main biologically active form is Ang II. Hypotension, hypovolemia, hemorrhage and osmotic challenge are known to activate RAS in fish. Consecutive hemorrhage in aglomerular toadfish, decreases blood pressure and increases plasma renin activity (Nishimura *et al.*, 1979). Bailey and Randall (1981) also found a linear relationship between the amount of blood loss and plasma renin activity, demonstrating that RAS must be involved in volume and pressure restoration. In the eel, the plasma renin activity also elevates when it is transferred from freshwater to seawater (Henderson *et al.*, 1976) and in Atlantic salmon (Smith *et al.*, 1991). Such osmotic challenge greatly decreases the blood volume of the animal and thus results in a drop in blood pressure. This will eventually activate the RAS to restore the blood pressure, leading to an increase in plasma renin activity. On the contrary, when the eel is transferred

from seawater to freshwater, the plasma renin activity decreases greatly.

Ang II is a potent pressor substance and increases blood pressure by vasoconstriction. Injection of renin from extract of Stannius corpuscle in eel leads to an increase in blood pressure as the exogenous renin elevates the circulating angiotensin levels (Chester Jones *et al.*, 1966). Injection of Ang II causes an increase in blood pressure in trout (Gray and Brown, 1985; Bernier and Perry, 1999), eel (Nishimura *et al.*, 1978; Oudit and Butler, 1995), flounder (Perrott and Balment, 1990), bowfin (Butler *et al.*, 1995), cod (Platzack *et al.*, 1993), and Antarctic fish (Axelsson *et al.*, 1994).

There is a higher resting blood pressure in freshwater eel than in seawater eel (Chester Jones *et al.*, 1969; Tierney *et al.*, 1995). However, a higher plasma renin activity is observed in seawater eel compared to freshwater one (Henderson *et al.*, 1976). Infusion of captopril in seawater-adapted eel causes a decrease in blood pressure while the treatment has no effect on the resting blood pressure in freshwater eel (Tierney *et al.*, 1995). Though the resting blood pressure is lower in seawater eel, it does not necessarily mean the circulating renin or angiotensin levels is also lower. This indicates that the resting blood pressure does not truly represent the status of RAS of an animal. Moreover, the RAS is likely to be operating at an activated state in seawater than in freshwater fish.

Injection of vasodilators such as papaverine and sodium nitroprusside is known to activate the endogenous RAS as they cause hypotension. Nishimura *et al.* (1979) showed that papaverine administration produced a marked hypotension followed by gradual recovery toward normal blood pressure, resulting in an increase in plasma renin activity and subsequently higher plasma Ang II levels. This indicates that RAS is important in restoring blood pressure from hypotension and such phenomenon has been widely used in the investigation of dipsogenic effects of RAS in fish which will be discussed in detail in the next section.

Olson *et al.* (1994) found that vasoconstriction by Ang II occurred in microcirculation in fish. However, Ang II-induced vasoconstriction plays no significant role in branchial circulation compared to systemic circulation in fish. The authors also demonstrated tachyphylaxis in the pressor response induced by Ang II. While epinephrine is not tachyphylactic, independent pressor components influenced by Ang II must be present. In a study on Antarctic fish, *Pagothenia borchgrevinki*, Ang II produced consistent hypertension by systemic vasoconstriction. The hypertension induced by Ang II was accompanied by a marked bradycardia, which could be abolished by atropine. This is in contrast to the results obtained after adrenaline injection, and suggests the existence of a cholinergic vagal reflex in fish (Axelsson *et al.*, 1994). Neither ACE inhibitor enalapril nor α-adrenergic blocker

prazosin could abolish the pressor effect of Ang II (Platzack *et al.*, 1993). Blockade of α-adrenoreceptors before RAS activation by papaverine prevented the recovery of blood pressure and systemic vascular resistance (Bernier *et al.*, 1999b). The authors suggested that both the RAS and the humoral adrenergic system participated in the acute hypotensive stress. Although it seems that the mechanism of increase in blood pressure by Ang II was related to adrenergic system, Bernier *et al.* (1999a) demonstrated that α-adrenergic blockage by prazosin had different effects on the restoration of blood pressure in response to Ang II between trout and eel. They also suggested that the involvement of adrenergic system in the pressure regulation depended considerably on the species. Nishimura *et al.* (1994) has shown that there are 3 types of angiotensin receptors at 3 locations on or near the blood vessel of fowl. They are on the adrenergic nerve ending, endothelium and smooth muscle [Fig. 3.2 (p.52)]. Adrenergic blockade can prevent catecholamine secretion while has no effects on other contractile machinery which is influenced by angiotensin receptors. Similar study has not yet been carried out in fish, but if the same model was present in fish, we might be able to explain the phenomenon that adrenergic blockade partially blocked the recovery of blood pressure during hypotension. In the evolutionary perspective, RAS acts on microcirculation in primitive vertebrates such as fish as an "anti-drop regulator" of blood pressure. Only through later evolution, this system

has been integrated into the variety of cardiac and nonvascular smooth muscle control system in mammal (Olson *et al.*, 1994).

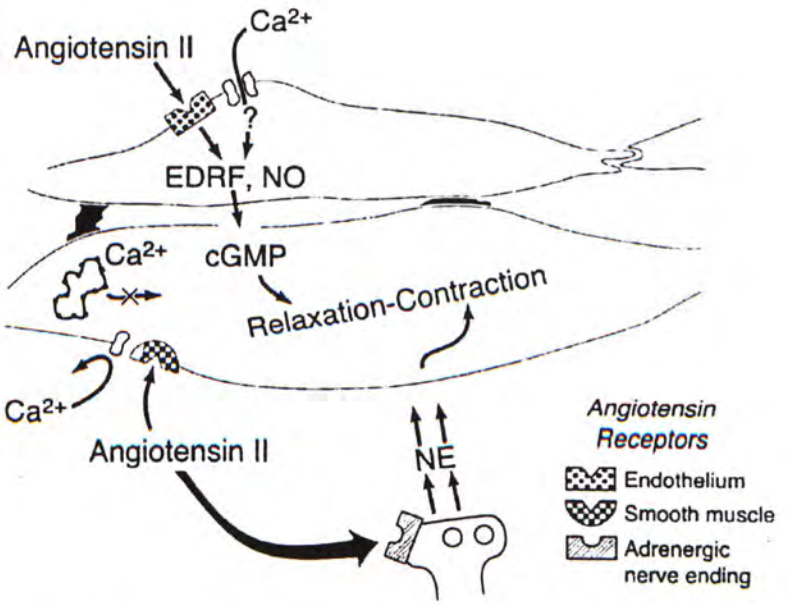

Fig. 3.2. Angiotensin receptors at 3 locations on or near blood vessel of fowl; endothelium, vascular smooth muscle, adrenergic nerve endings. Ang II exerts dual action in the vascular system: (1) vasocontraction via the release of catecholamines and (2) relaxation via endothelial angiotensin receptor. EDRF, endothelium-derived relaxing factor; NO, nitric oxide; NE, norepinephrine. (From Nishimura *et al.*, 1994)

3.1.3 RAS and Drinking

The role of RAS in the control of drinking in fish and other vertebrates has been well-documented (Kobayashi and Takei, 1996). It is known that Ang II, the biological active form, is a potent dipsogen in animals. Ang II induces drinking in reptiles (Fitzsimons and Kaufman, 1977), birds (Takei, 1977; Kobayashi, 1981) and mammals (Fitzsimons and Simons, 1968), and increases water-absorption of the skin in amphibians (Erspamer *et al.*, 1979). In bony fish, Ang II is dipsogenic in many common experimental models such as rainbow trout (Fuentes and Eddy, 1998), Atlantic salmon (Fuentes and Eddy, 1997), European eel (Tierney *et al.*, 1993), killifish (Malvin *et al.*, 1980), flounder (Carrick and Balment, 1983), and goldfish (Kobayashi, *et al.*, 1983). Besides direct injection of Ang II, many investigators have attempted to activate the endogenous RAS using vasodilators such as papaverine and sodium nitroprusside to stimulate drinking in fish (Balment and Carrick, 1985; Fuentes and Eddy, 1998; Fuentes *et al.*, 1996). Such experiments did provide strong evidence that RAS is involved in the regulation of drinking rate of fish.

Kobayashi *et al.* (1983) examined 37 species of bony fish and found that Ang II generally does not stimulate drinking in those fishes which live exclusively either in freshwater or seawater because osmotic challenges are rare in these environments. Only those species that are subjected to osmotic challenges such as those living in

brackish water or in tide pools increase their drinking rate in response to Ang II. Fishes inhabiting only seawater or freshwater are exposed to osmotically constant environment and thus regulation of drinking rate become relatively unimportant. Carnivorous birds also do not increase drinking rate in response to Ang II as they obtain water from their preys (Kobayashi *et al.*, 1979). Kobayashi and Takei (1996) suggested that in species which drink only a little water in nature or seldomly regulate their drinking rate, the angiotensin-thirst mechanism would become attenuated or be lost during evolution. However, exceptional cases have been recorded in Richardson's dragonet (*Callionymus richardsoni*) and redfin velvetfish (*Hypodytes rubripinnis*) as an inhibitory effect of Ang II on drinking has been observed (Kobayashi *et al.*, 1983). The mechanism of such an inhibition is still unknown and more exceptional cases are expected.

Injection of ACE inhibitors such as captopril and enalapril lowers the basal drinking rate in seawater-adapted killifish (Malvin *et al.*, 1980), Atlantic salmon (Fuentes and Eddy, 1997), flounder (Balment and Carrick, 1985), and European eel (Tierney *et al.*, 1993). However, captopril injection did not alter the drinking rate in European eel adapted to freshwater (Tierney *et al.*, 1993). Furthermore, it is shown that captopril may enhance water intake in freshwater-adapted goldfish (Okawara and Kobayashi, 1988). The authors explained that after captopril inhibition, Ang I was

accumulating in the plasma and there was an increase in plasma renin activity (Schiffrin *et al.*, 1981). After the effect of captopril became attenuated as time passed, the pooled Ang I in the circulation was quickly converted into Ang II which then led to an increase in drinking rate (Schiffrin and Genest, 1982). Though the effects of Ang II and captopril on drinking rate vary considerably among teleosts, it is generally accepted that RAS is more important in the regulation of drinking rate in seawater fish than freshwater fish. Attention also has been drawn towards the role of RAS in the inhabitation of seawater from freshwater (Balment and Carrick, 1985). It was suggested that when euryhaline fish inhabit seawater from freshwater, RAS is activated to stimulate drinking to compensate for the water loss, and to elevate blood pressure in response to hypovolemia.

3.1.4 RAS and cortisol

Exogenous renin and Ang II increase not only blood pressure, but also circulating levels of adrenocorticotrophic hormone (ACTH) and cortisol in freshwater eel (Henderson *et al.*, 1976). Injection of renin increases the circulating cortisol level in intact eel but not in hypophysectomized eel. Administration of Ang II and papaverine causes an increase in cortisol level in flounder (Perrott and Balment, 1990). The same study also showed captopril blockade prior to papaverine administration abolished the increase in circulating cortisol. Plasma cortisol level also has a similar

rise following induced hypotension and hemorrhage in flounder (Carrick and Balment, 1984). When the eels are transferred from freshwater to seawater, the elevation in plasma cortisol level is blocked by administration of captopril (Kenyon *et al.*, 1985). In goldfish pituitary cell column, both Ang I and Ang II stimulate the release of ACTH (Weld and Fryer, 1987). The authors suggested that the RAS activates the whole ACTH-cortisol axis and steroidogenesis in fish and this phenomenon appeared early in the evolution of vertebrate pituitary. As a whole, these results suggest that activation of RAS, both exogenous and endogenous, increases steroidogenesis and cortisol secretion in fish. Therefore, the RAS may be of physiological significance in the control of cortisol secretion.

It is also found that Ang II stimulates prolactin release from the rostral pars distalis of tilapia (Grau *et al.*, 1984), while prolactin is known to be important in regulating membrane permeability in freshwater fish (Pickford and Phillips, 1959; Morley *et al.*, 1981). Also, replacement therapy in hypophysectomized killifish showed that prolactin is essential for survival in freshwater. This is in contrast to the view that RAS is solely working in seawater, so further investigations are needed to confirm this point. Cortisol in teleostean fish plays an important role in osmoregulation in a hyperosmotic environment by promoting the extrusion of sodium across the gills and by increasing water permeability of the intestine (Nishimura,

1980). It seems that RAS has widespread effects on water and electrolyte balance in fish, both in freshwater and seawater. As angiotensin levels govern cortisol and prolactin secretion, RAS may serve to maintain salt and water balance.

Cortisol was also found to change the morphology of chloride cells in fish. Chloride cells have different morphology and completely opposite function in seawater and freshwater. In freshwater, the chloride cells have a protruding apical surface and have plenty of microvilli on the surface. This maximizes the apical surface area for ion uptake in freshwater fish. In seawater chloride cells, the apical surface is sunken in shape with an apical pit and without microvilli. This minimizes the disturbance of the micro-environment inside the apical pit in order to avoid water loss. The above-mentioned chloride cell morphologies are also identified in seabream from hypersaline to freshwater (Kelly *et al.*, 1999a). Exogenous cortisol administration of cortisol increases chloride cells size (Madsen, 1990), stimulates the development of tubular system (Doyle and Epstein, 1972) and also increases salinity tolerance (Epstein *et al.*, 1971). In both directions of adaptation, an elevation of plasma cortisol is observed in fish, but plasma cortisol levels are unaltered in long-term salinity adaptation (Kelly and Woo, 1999b). This indicates that the cortisol boost during adaptation may play an important role in reorganizing the body structure especially the chloride cell morphology and function.

Cortisol was shown to stimulate gill Na^+/K^+-ATPase activity in killifish (Pickford *et al.*, 1970), eel (Epstein *et al.*, 1971), tilapia (Dange, 1986), and salmon (Richman and Zaugg, 1987), etc. and is often referred as the "seawater-adapting hormone". Combining the effects of RAS in the control of cortisol secretion and its own function on Na^+/K^+-ATPase activity, it can be concluded that RAS must be vital in seawater inhabitation, and the RAS should deserve the name as "seawater-adapting system". Beside the indirect effect of cortisol on Na^+/K^+-ATPase, it is recently shown that Ang II has a direct stimulating effect on the activity of this enzyme in both branchial and renal tissue in eel (Marsigliante *et al.*, 1997; 2000). In mammal, captopril treatment was also found to inhibit the Na^+/K^+-ATPase activity in retina of rat (Ottlecz and Bensaoula, 1996).

3.1.5 RAS and kidney

Ang II appears to exert a direct intrarenal antidiuretic effect as it reduced glomerular filtration rate (GFR) and urine formation in freshwater trout (Brown *et al.*, 1980). Nishimura and Imai (1982) also found an increase in blood pressure, inulin clearance, urine flow and sodium excretion after Ang II infusion. On the other hand, captopril was found to be a diuretic drug in fish as it increases both the GFR and urine volume of both seawater and freshwater trout (Kenyon *et al.*, 1985). This implied that Ang II is an important substance in conserving water. This property is highly

relevant to seawater survivial as the fish is constantly losing water. Gray and Brown (1984) suggested that these changes might be related to seawater adaptation of the fish when they migrate from hyposmotic to hyperosmotic media. It was hypothesized that Ang II constricted both the afferent and efferent glomerular arterioles, resulting in decrease in GFR and subsequently urine formation (Sokabe *et al.*, 1973). However, Christensen *et al.* (1989b) found that Ang II receptors were mainly located at the afferent arterioles, and therefore suggested that the RAS in fish mainly acts on the afferent side of the glomerulus. The affinity of Ang II to the receptors in isolated glomeruli is high in both freshwater and seawater trout (Cobb and Brown, 1994). This may imply that RAS exerts a rapid effect on the glomeruli for osmotic balance in both freshwater and seawater. Furthermore, *in vitro* incubation of kidney slices with Ang II inhibited renin secretion, suggesting a negative feedback mechanism of the renin secretion within kidney might be present (Bailey and Randall, 1981). A more recent study by Brown *et al.* (2000) demonstrated that a complete intrarenal RAS is present in trout kidney and its function can be independent from the system RAS.

3.1.6 Summary

The RAS is conserved throughout vertebrate evolution and is important for hydro-mineral balance. There is increasing evidence to show that the RAS is an important system for seawater adaptation in fish. When fish inhabit seawater from

freshwater or hyposmotic media, RAS is activated and leads to a multitude of physiological adjustments. The blood pressure is restored by the pressor effect of Ang II due to hypovolemia, as water is lost to the external environment. The drinking rate is stimulated by Ang II to compensate for water loss. Activated RAS leads to an increase in circulating cortisol by stimulating the release of ACTH from the pituitary and the cortisol elevates the Na^+/K^+-ATPase activity in gill, kidney and intestine. Ang II also directly stimulates the Na^+/K^+-ATPase activity in gill and kidney while the increase in the activity of this enzyme is vital for the fish to inhabit seawater. Increased cortisol level also promotes the morphological and functional modification of chloride cells to increase salinity tolerance. Ang II is an antidiuretic factor that lowers both GFR and urine flow and helps the fish to conserve water in hyperosmotic media. In a nutshell, the RAS is involved in the control of a wide range of osmoregulatory parameters including cardiovascular properties, chloride cell morphology, renal function, drinking appetite and hormonal status, all of which are vital for the fish to survive in fluctuating salinities.

3.2 Materials and Methods

3.2.1 Experimental animals

Silver seabream (*Sparus sarba*) were obtained from local sea cage. The culture condition was the same as described in section 2.2

3.2.2 Salinity adaptation

All the fish were adapted to specified salinity (6, 12, 33 ppt) for at least 4 weeks before any experiment was done. The adaptation condition was the same as described in section 2.2

3.2.3 Drinking rate measurement

Drinking rate was measured as described in section 2.2. Drinking rate was measured in fish adapted to (1) 6, 12 and 33 ppt injected with saline, (2) 6, 12, 33 ppt injected with captopril (10 mg/kg), (3) 6, 12, 33 ppt injected with Ang II (10 μg/fish). The effect of various doses of Ang II (0.001, 0.01, 0.1 mg/fish) and SNP (1, 10, 100 nmol/kg) on drinking rate of 33 ppt-adapted fish was also performed. Drinking rate was also measured in 6ppt-adapted fish injected with SNP (0.1, 1 nmol/kg). All injections were performed intraperitoneally and vehicle (0.8% NaCl) injected was less than 0.2 ml.

3.2.4 Determination of angiotensin converting enzyme activity

The angiotensin converting enzyme (ACE) activity was determined according to

Cushman and Cheung (1971). Gill tissue from fish adapted to 6, 12, 33 and 50 ppt was obtained and stored at –70 °C. Tissue was homogenized in EDTA-free buffer (100 mM KH_2PO_4, 300 mM NaCl). Hippuryl-L-histidyl-L-leucine (HHL) was used as an artificial substrate for ACE. HHL reagent (5mM in buffer) was added to the homogenate and incubated 30 min with shaking. The reaction was stopped by adding 1N HCl and the hippuric acid formed was extracted using 1 : 1 ethyl acetate. The ethyl acetate was then evaporated at 120 °C and the residue was redissolved in 1 ml distilled water. The amount of hippuric acid was determined spectrophotometrically at 228 nm. One unit of ACE activity is defined as the amount of enzyme catalyzing the formation of 1μmole of hippuric acid from HHL in 1 min. at 25 °C.

3.2.5 Blood pressure experiment

The afferent branchial artery was cannulated as described before previously and blood pressure was measure by the same method as outlined in section 2.2. A three-way stopcock was connected between the transducer and the cannula for drug administration and to flush the cannula. All drugs were administrated as bolus injection through the cannula in less than 0.2 ml physiological saline.

Blood pressure was measured in (1) 6 and 33 ppt fish injected with physiological saline, (2) 6 and 33 ppt fish injected with captopril (0.20 mg/kg), 6 and 33 ppt fish

with bolus injection of Ang II (0.1 μg/kg). Blood pressure was also measured in fish with abrupt salinity transfer from 33 to 6 ppt and *vice versa* with captopril blockade.

3.2.6 Statistical analysis

All data are expressed as mean values ± SEM. Data from various groups were subjected to a one-way ANOVA to test for significance followed by a Tukey comparison test to delineate significance between groups. Independent comparisons between treatment and control were subjected to Student's t test with $P<0.05$ to delineate significance between the two groups.

3.3 Results

3.3.1 Drinking rate

The drinking rate of fish injected with saline was found to be similar to those intact fish, suggesting that handling stress would not alter the drinking rate of fish. When 6 ppt fish were injected with Ang II (0.01 mg/fish), there was a 4-fold increase in drinking rate, a level that was the double of the drinking rate of 33 ppt fish [Fig. 3.3 (p.66)]. The selected dose of Ang II injection was comparable to those used by Kobayashi *et al.* (1983). Administration of the same dose of Ang II doubled the drinking rate of 12 ppt [Fig. 3.4 (p.66)] fish but lowered the drinking rate of 33 ppt fish [Fig. 3.5 (p.67)]. Captopril (10 mg/kg), an ACE inhibitor, did not affect the drinking rate of 6 and 12 ppt fish but lowered the drinking rate of 33 ppt fish. The dose response relationship of Ang II on 33 ppt fish is shown in Fig. 3.6 (p.67), while the lower dose of Ang II did not affect the drinking rate compared with the control group, the higher dose inhibited the drinking rate of 33 ppt fish. Such inhibitory effect of Ang II on drinking was rarely observed and only 2 records (*Callionymus richardsoni* and *Hypodytes rubripinnis*) had been made by Kobayashi *et al.* (1983). Therefore, instead of using exogenous Ang II as a stimulator of RAS in seabream, a vasodilator, SNP (sodium nitroprusside) was also used to activate RAS endogenously. It was found that after injection of 100 nmol/kg SNP (the same dose used by Fuentes

et al. (1996) on juvenile salmon), the seabream struggled vigorously and lost balance, indicating that this dosage was too high for seabream. No struggling was observed until the dose was lowered to 1 nmol/kg and the drinking rate of seabream after such treatment dropped dramatically compared with the saline control group [Fig. 3.7 (p.68)]. When the 6 ppt fish was injected with 1 nmol/kg SNP, struggling was observed but when the dose was lowered to 0.1 nmol/kg, no struggling was observed and the drinking rate was double compared to the saline control group [Fig. 3.8 (p.68)].

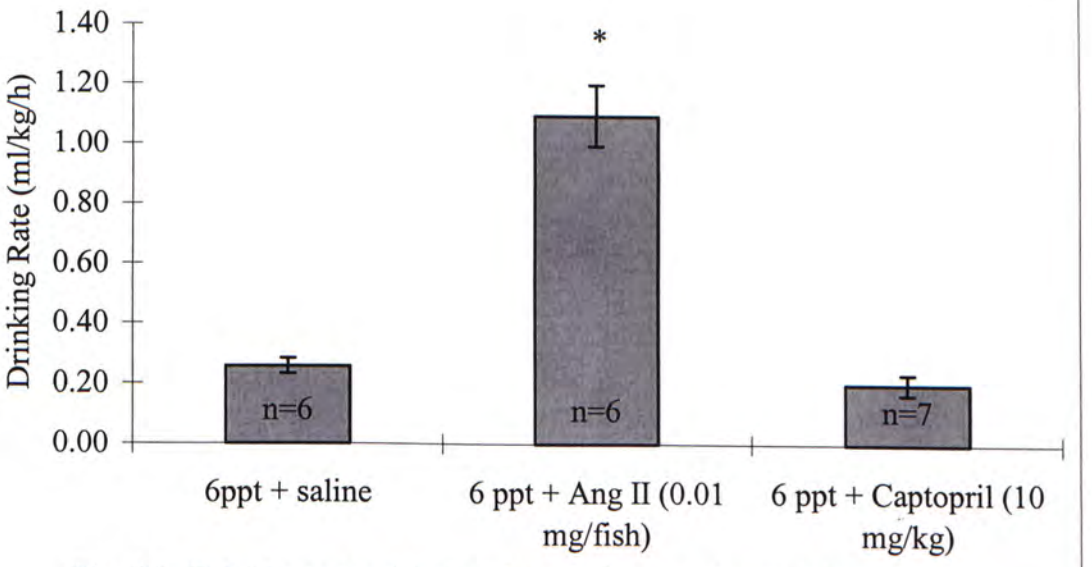

Fig. 3.3. Drinking rate of 6 ppt-adapted silver seabream injected with saline, Ang II and captopril. *Denote significant difference ($P<0.05$) with the saline-control group

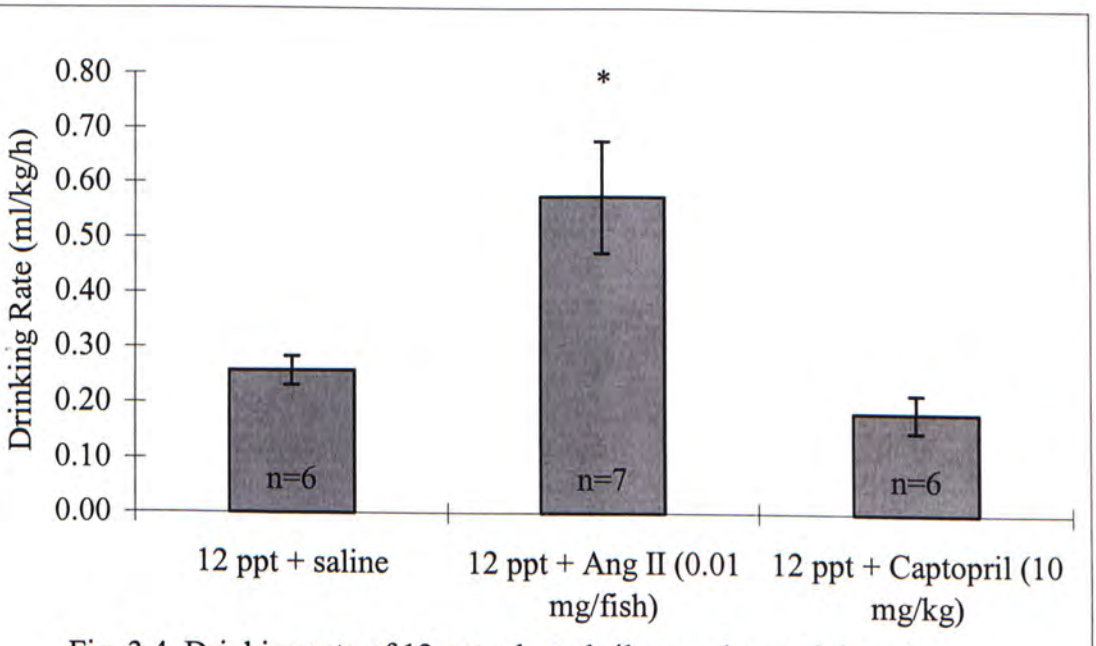

Fig. 3.4. Drinking rate of 12 ppt-adapted silver seabream injected with saline, Ang II and captopril. *Denote significant difference ($P<0.05$) with the saline-control group

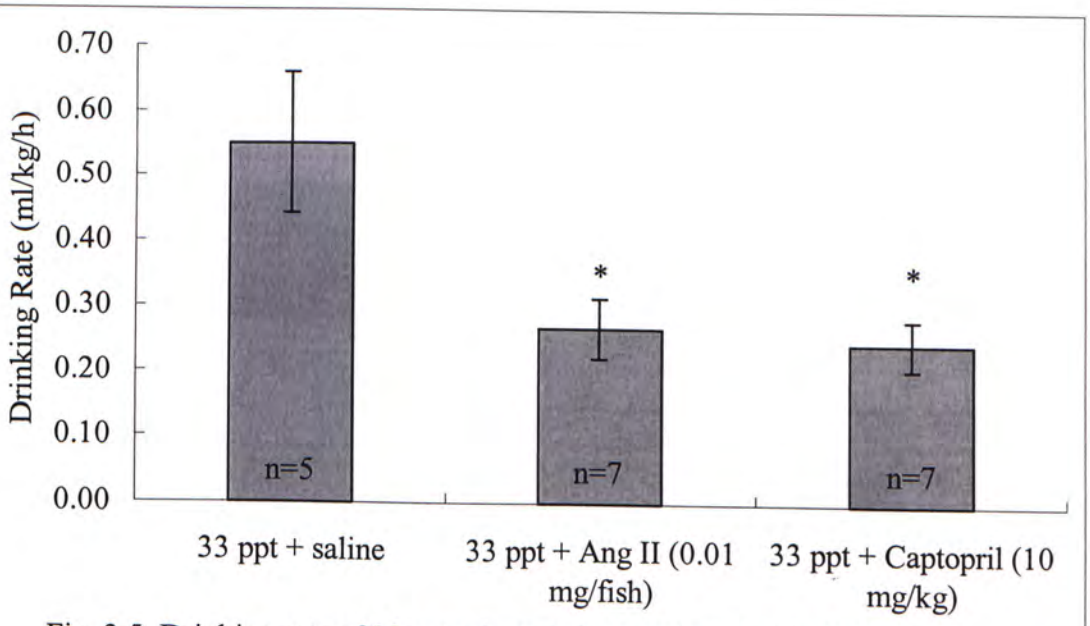

Fig. 3.5. Drinking rate of 33 ppt-adapted silver seabream injected with saline, Ang II and captopril. *Denote significant difference ($P<0.05$) with the saline-control group

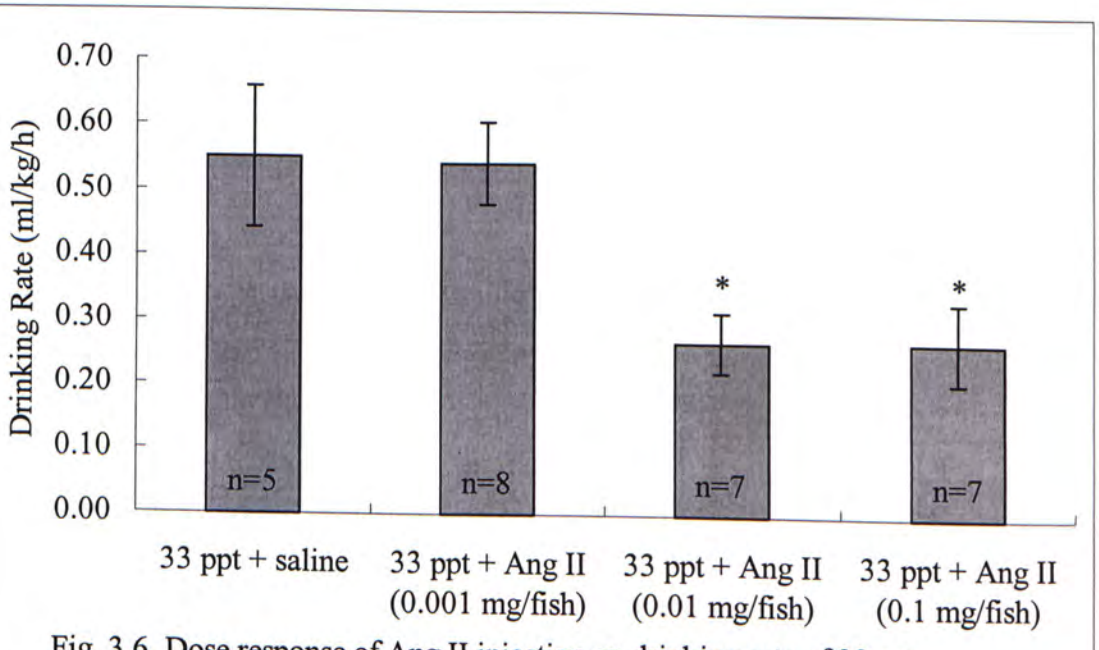

Fig. 3.6. Dose response of Ang II injection on drinking rate of 33 ppt-adapted silver seabream. *Denote significant difference ($P<0.05$) with the saline-control group

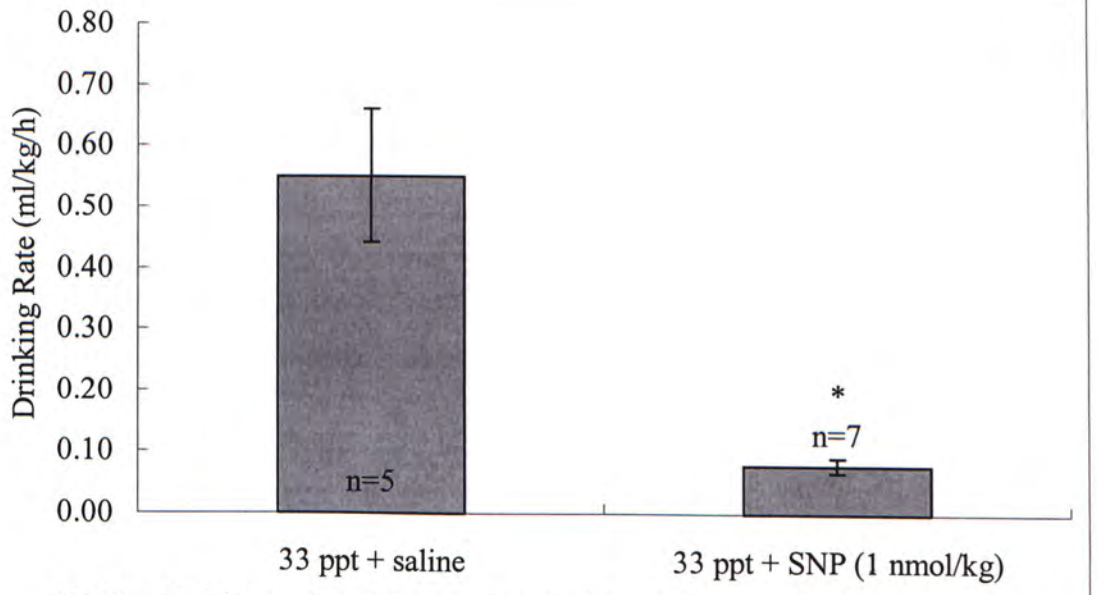

Fig. 3.7. Drinking rate of 33 ppt-adapted silver seabream injected with saline and SNP. *Denote significant difference ($P<0.05$) with the saline-control group

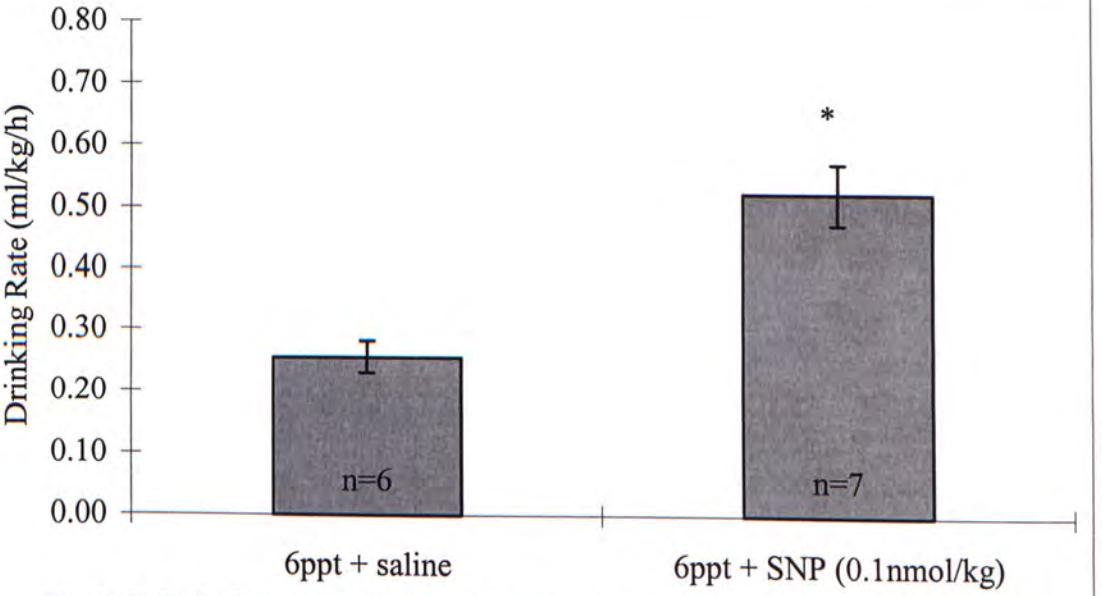

Fig. 3.8. Drinking rate of 6 ppt-adapted silver seabream injected with saline and SNP. *Denote significant difference ($P<0.05$) with the saline-control group.

3.3.2 ACE activity

The activity of brachial ACE of seabream adapted to different salinities is presented in Fig. 3.9 (p.70). The ACE activity was highest in 33 ppt fish, but no significant difference was observed among 6, 12 and 50 ppt fish. A modification was made in the experimental setup compared to that of Galardy *et al.* (1984) who studied rainbow trout. The temperature used in the present experiment was 25 °C while the rainbow trout experiment was performed at 37 °C. The upper lethal temperature of rainbow trout is around 25 °C, so the enzymatic activity measured by Galardy *et al.* (1984) must have been distorted by unfavorable temperature. Therefore, a lower temperature was chosen in the present experiment because fish are mostly ectothermic animals while high temperature may cause enzyme denaturation and high enzyme activity outside the physiological ranges.

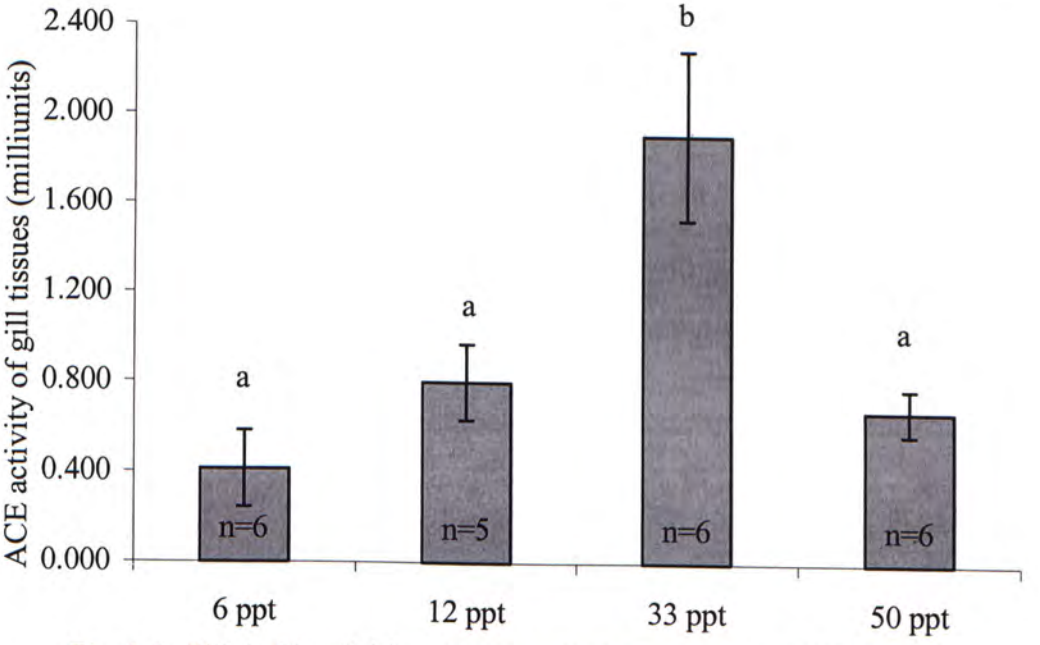

Fig. 3.9. Gill ACE activities of silver seabream adapted to different salinities. Groups with different letters are significantly different from each other($P<0.05$).

3.3.3 Blood pressure

Blood pressure of silver seabream injected with physiological saline showed no significant difference with the pre-injection value. Blood pressure of 33 ppt and 6 ppt seabream was significantly lowered by administration of captopril directly into the blood stream. This showed that in both hyperosmotic and hyposmotic media, RAS was at an activated state and responsible for maintaining the basal blood pressure. While captopril also lowered the blood pressure in 6 ppt seabream, an observation that differed from the results obtained in freshwater eels, in which captopril did not have any effect on resting blood pressure (Tierney *et al.*, 1995). However, comparing the level of blood pressure lowering, the same dosage of captopril imposed a larger drop in blood pressure in 33 ppt seabream than the 6 ppt fish. This showed that the status of RAS in 6 ppt fish was at a less activated state. Comparing the level of inhibition of captopril in the blood pressure of 33 ppt and 6 ppt fish, RAS seemed to play a more significant role in seawater adaptation. When the captopril-blocked seabream was abruptly transferred from 33 ppt to 6 ppt or *vice versa*, the blood pressure did not show significant changes when compared with the pre-transfer values. Moreover, the mode of blood pressure maintenance was similar to those observed in captopril-free fish as presented in section 2.3.4.

When the fish was injected with Ang II, the blood pressure increased

significantly in both 33 ppt and 6 ppt fish [Fig. 3.12 (p.75) ; Fig. 3.13 (p.76)]. This showed that angiotensin is vasopressive in seabream. The pattern of increment in blood pressure by Ang II in the 2 salinity adapted groups had no significant difference. The vasopressive effect of Ang II disappeared within 15 min after the injection, which indicated that the metabolism of the peptide is fast and the effect is instant and potent.

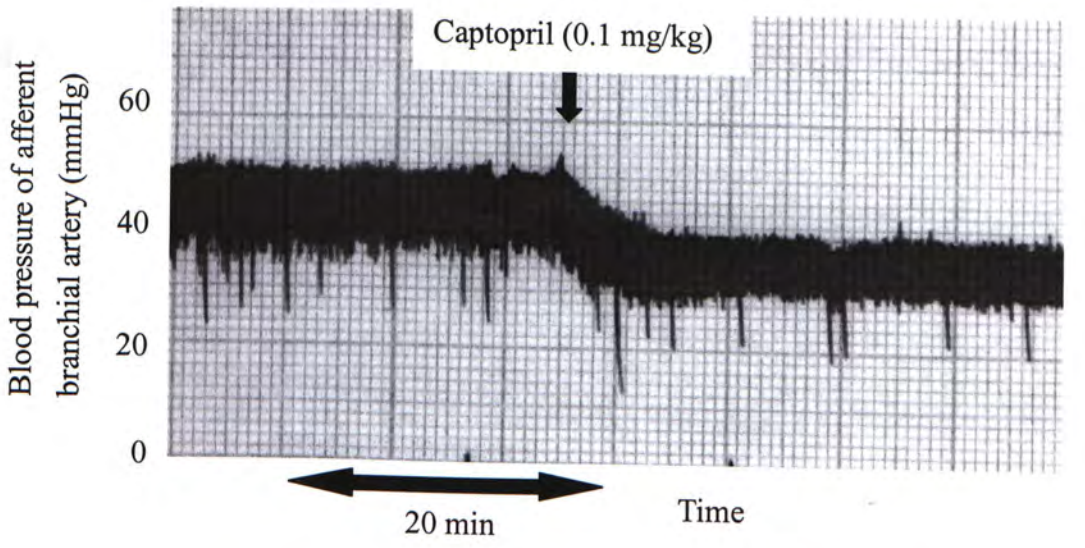

Fig. 3.10a. Original tracing of afferent branchial blood pressure of 33 ppt silver seabream injected with captopril (0.1 mg/kg). (typical example)

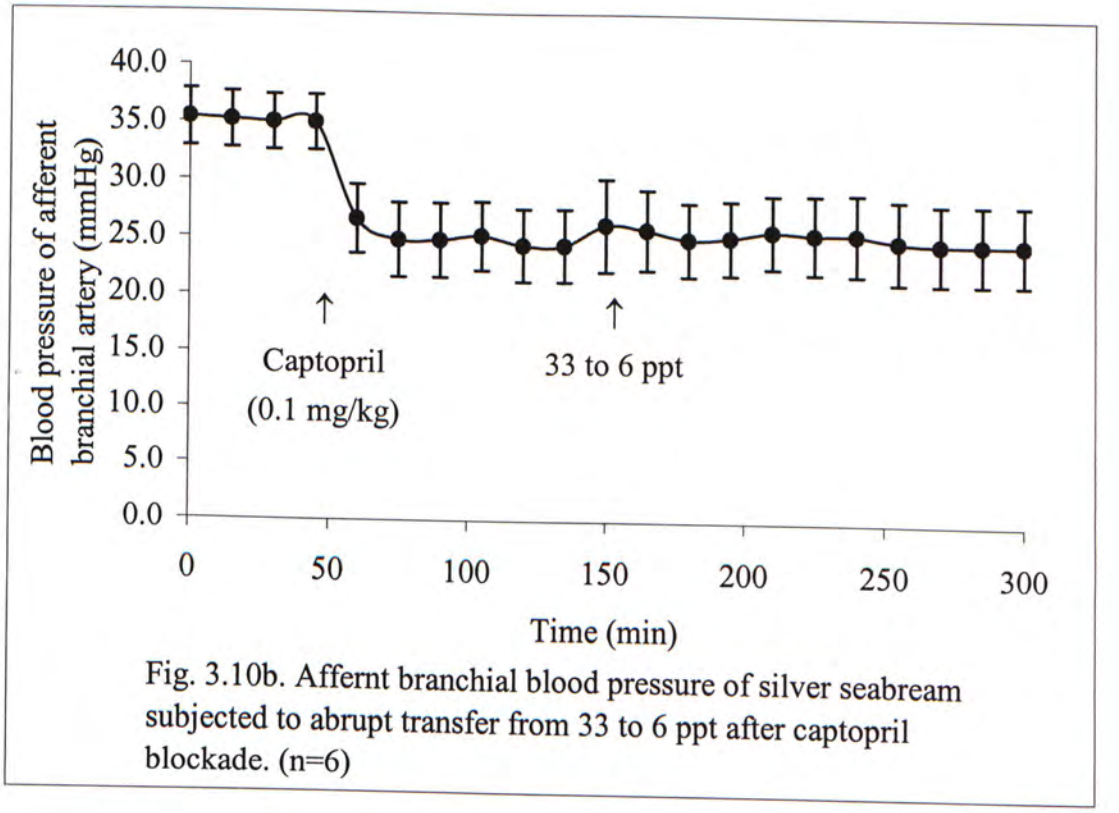

Fig. 3.10b. Affernt branchial blood pressure of silver seabream subjected to abrupt transfer from 33 to 6 ppt after captopril blockade. (n=6)

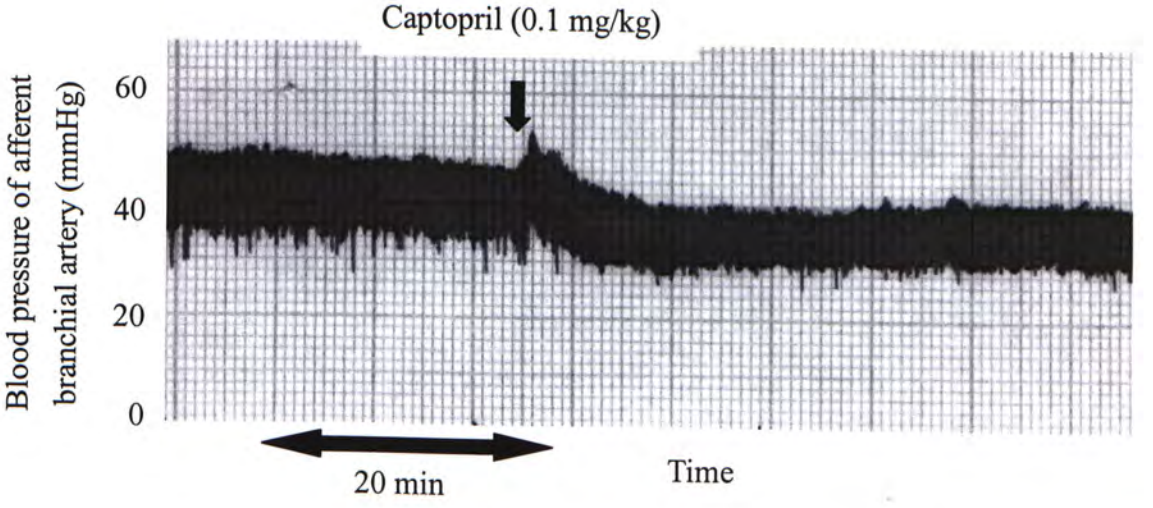

Fig. 3.11a. Original tracing of afferent branchial blood pressure of 6 ppt silver seabream injected with captopril (0.1 mg/kg). (typical example)

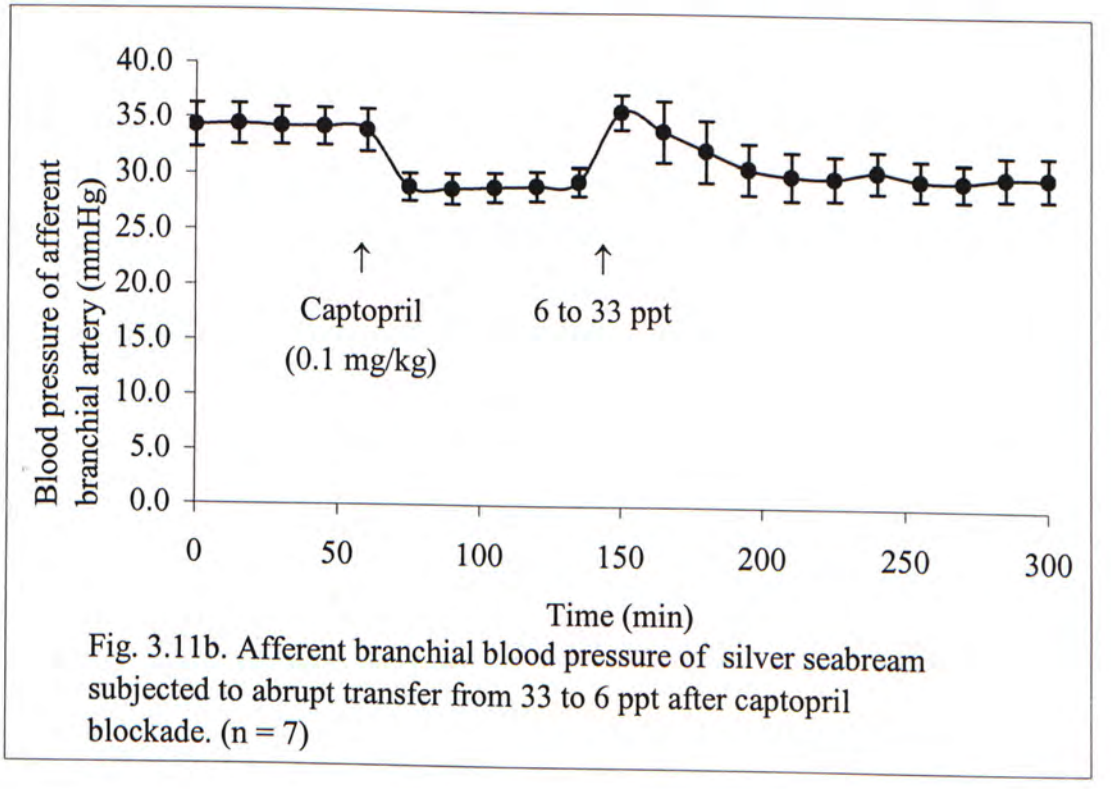

Fig. 3.11b. Afferent branchial blood pressure of silver seabream subjected to abrupt transfer from 33 to 6 ppt after captopril blockade. (n = 7)

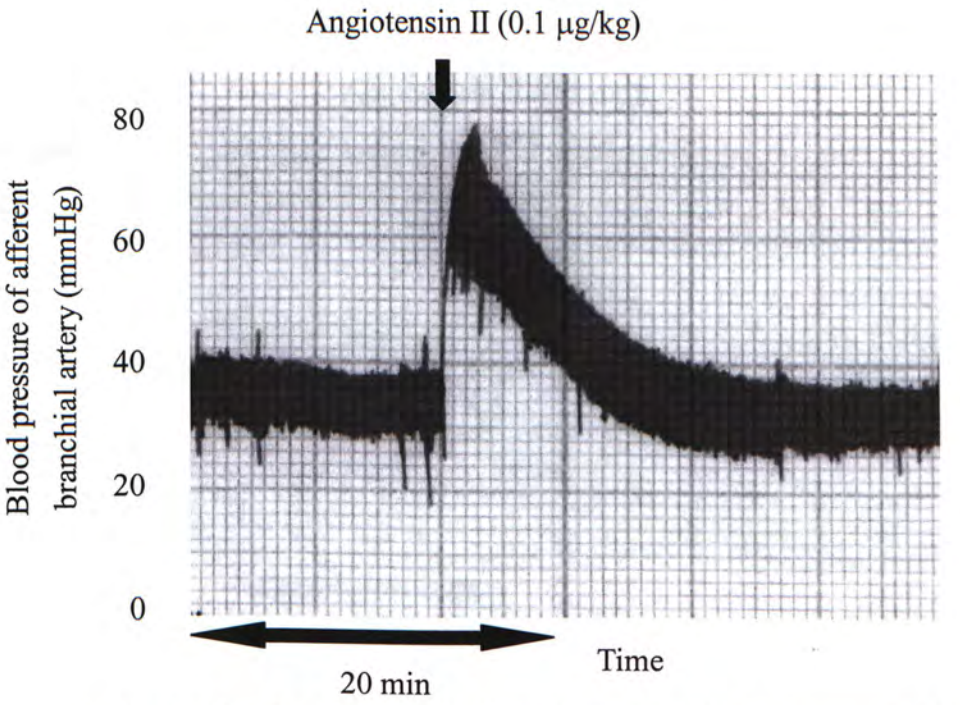

Fig. 3.12a. Original tracing of afferent branchial blood pressure of 33 ppt sliver seabream injected with angiotensin II (0.1 μg/kg). (typical example)

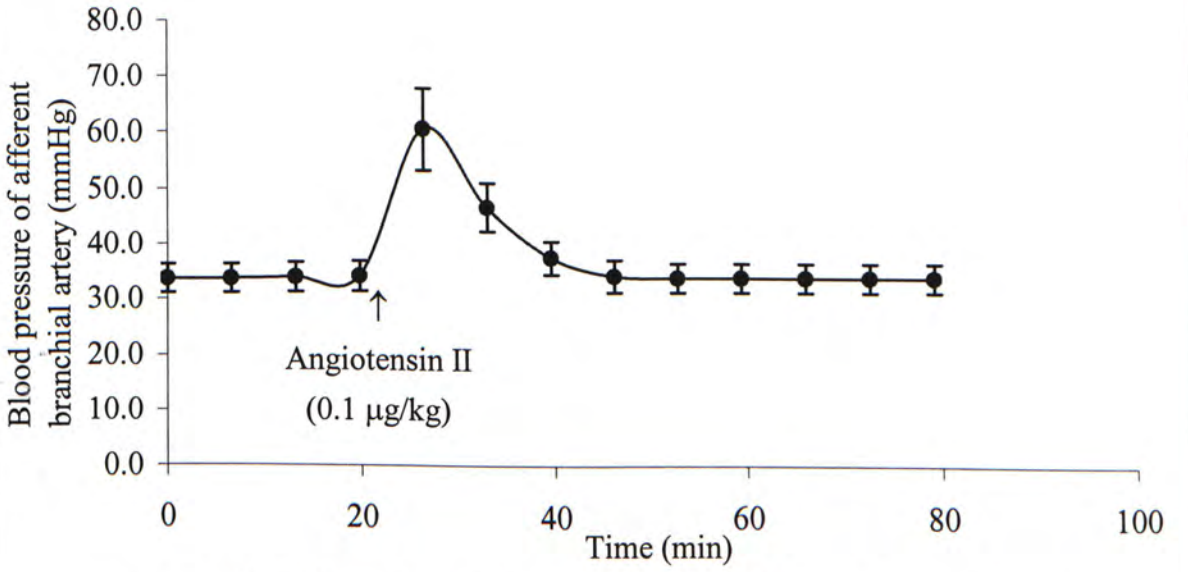

Fig. 3.12b. Afferent branchial blood pressure of 33 ppt silver seabream injected with Ang II. (n = 6)

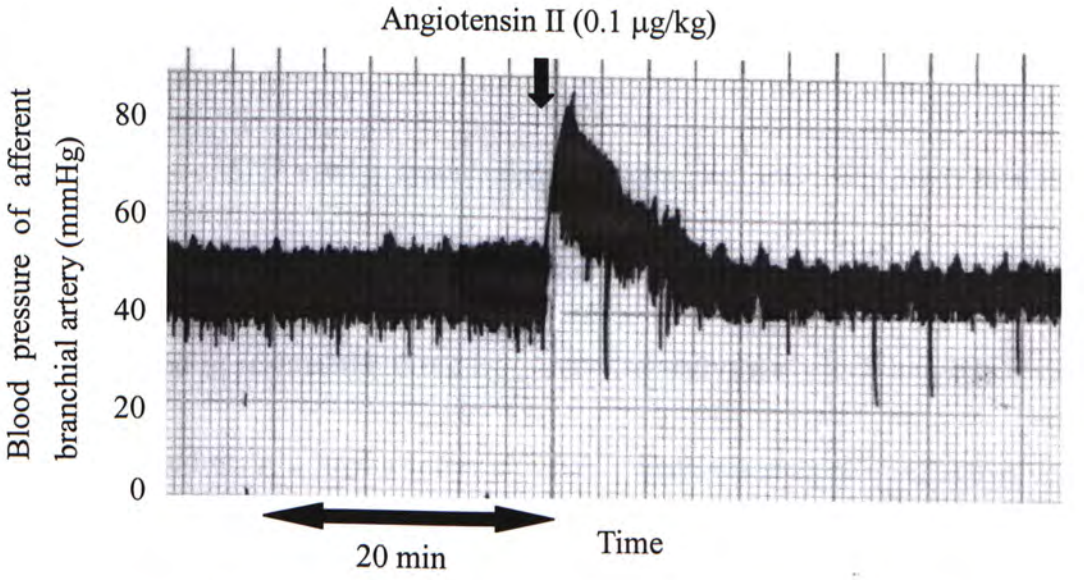

Fig. 3.13a. Original tracing of afferent branchial blood pressure of 6 ppt silver seabream injected with angiotensin II (0.1 μg/kg). (typical example)

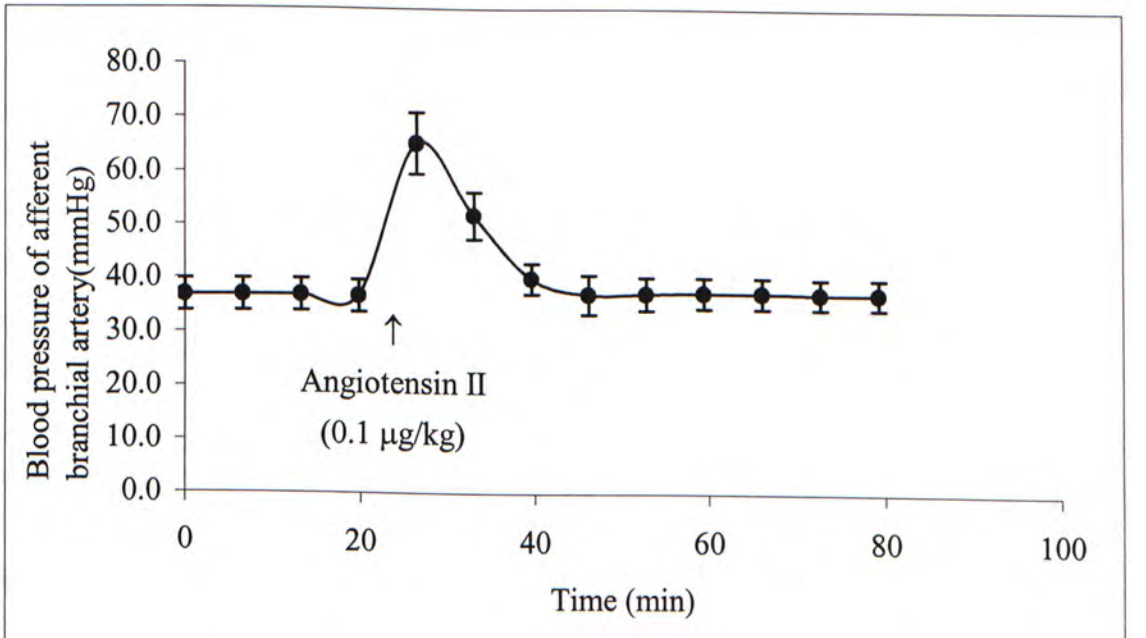

Fig. 3.13b. Afferent branchial blood pressure of 6 ppt silver seabream injected with Ang II. (n = 6)

3.4. Discussion

3.4.1 Drinking rate

The dosage of SNP used in seabream (0.1 - 1 nmol/kg) was much lower than that used in juvenile salmon (100 nmol/kg; Fuentes *et al.*, 1996). Moreover, the dosage of captopril used in seabream (10 mg/kg intraperitoneally) was much lower than that used in trout and eel (72 mg/kg intravenously). Such differences in effective dose may be due to species differences in receptor abundance and responsiveness of motor organs.

It is well known that Ang II is a dipsogen and can stimulate drinking in vertebrates in general. Ang II was also shown to stimulate drinking in both seawater and freshwater fishes (Kobayashi *et al.*, 1983). In the present study, Ang II increased the drinking rate in both 6 and 12 ppt seabream. This phenomenon implies that hyposmotic adapted seabream behaves as a typical freshwater fish. Moreover, the level of stimulation in drinking in 6 ppt fish was higher than that of 12 ppt fish. This indicated that 6 ppt fish is more sensitive to angiotensin than 12 ppt fish in drinking response. From a functional perspective, the difference in sensitivity allows the 6 ppt fish to cope with the hyperosmotic challenge more rapidly. Drinking rate was not significantly altered in 6 and 12 ppt fish under captopril treatment, this may indicate that RAS is not in an activated state in both salinities. In 33 ppt fish,

captopril treatment lowered the drinking rate and this may imply that RAS is activated in contrast to the 6 and 12 ppt fish. In other words, RAS plays a role to maintain the basal drinking rate that is vital in seawater fish. These observations are comparable to those obtained in trout (Fuentes and Eddy, 1998), salmon (Fuentes and Eddy, 1997), eel (Tierney *et al.*, 1993), flounder (Carrick and Balment, 1983), goldfish (Kobayashi *et al.*, 1983), and killifish (Malvin *et al.*, 1980). However, exogenous Ang II caused a drop in drinking rate in 33 ppt seabream, an observation which is completely in contrast to the classical dipsogenic property of Ang II. The dose-response relationship shown in Fig. 3.4 (p.67) also indicated that higher dose did not stimulate but inhibited the drinking of seawater seabream. An attempt had been made to activate the endogenous RAS by a vasodilator, SNP to confirm the inhibitory effect of Ang II. Same inhibitory result was obtained [see Fig. 3.5 (p.68)] and the drinking rate was lowered by the activation of endogenous RAS. When the 6 ppt fish was injected with SNP, drinking rate increased significantly, indicating that endogenous activation of RAS could increase circulating Ang II level and led to increase in drinking rate in hyposmotic seabream [see Fig. 3.6 (p.68)].

Kobayashi *et al.* (1983) also reported 2 species that exhibited an inhibitory response in drinking after Ang II injection. They are the *Callionymus richardsoni* (Richardson dragonets) and *Hypodytes rubripinnis* (redfin velvetfish), which are some

sandy benthic fish on the continental shelf. In the same study, the authors also reported an unresponsive species, *Acanthopagrus schlegeli* (black porgy), which is closely related to the experimental model (*Sparus sarba*) in the present study. The mechanism of the inhibitory effect of Ang II on drinking rate in seawater fish has not yet been studied. As most of fish physiological investigations depend on several experimental models such as trout, eel, flounder, salmon, etc., generalization made by these studies may not be representative. A survey study like that of Kobayashi *et al.* (1983) would reveal a relatively more representative situation. For example, some teleost species are actually unresponsive to Ang II in drinking behavior. In addition to the 2 species reported by the authors, the present study provides another example (seabream) that possesses inhibitory effect of Ang II on drinking and more exceptions are expected in the future. Furthermore, the role of RAS seems to vary in the same species at different salinities. However, the importance of the different functions is still an opened area for investigation.

As a bimodal relationship between Ang II and drinking behavior of seabream adapted to different salinities was observed, it was obvious that generalization cannot be made so easily on the role of RAS in fishes. It was interesting that captopril inhibition lowered the drinking rate of 33 ppt fish, indicating that RAS should play a role in the regulation of basal drinking rate of fish. However, the route of captopril

inhibition is on the inhibition of conversion of Ang I to Ang II endogenously, the effects of captopril on other system should not be neglected. Inhibition of ACE not only lowers the circulating Ang II levels, but also increase the level of metabolic active bradykinin and kallidin, a component in the kallikrein-kinin system (KKS). ACE is also found to catalyze the degradation of active bradykinin and kallidin to inactive forms. As there are few studies on the KKS in fishes, the physiological significance of this system is not known yet (Olson, 1992). Furthermore, the effects of kinins in fishes are different from mammals as kinins are potent depressor and vasodilator in the later but have some pressor effect in trout (Lipke *et al.*, 1990) and eel (Chan and Chow, 1976). A recent study in cod also revealed that bradykinin is vasopressive and comparison of route of action of kinins between cod and mammals further suggested that KKS in fish is distinct from mammals (Platzack and Conlon, 1997). An impact finding in eel showed that captopril can lower the drinking rate, blood pressure and circulating Ang II in seawater eel, but lowering of Ang II by anti-Ang II serum did not affect the drinking rate and blood pressure (Takei and Tsuchida, 2000). As the drinking of eel was not inhibited by the immuno-neutralization of plasma Ang II, a treatment that lowered circulating Ang II levels, it seems that the level of Ang II has no direct effect on the drinking behavior in seawater eel. In contrast, homologous bradykinin, which probably increased in

plasma after captopril treatment, potently inhibited copious drinking in seawater eel (Takei, 2000). As seabream responded to Ang II injection by lowering in drinking rate, it is hypothesized that RAS activation in seawater seabream may somehow increase circulating kinins that override the effect of Ang II and inhibit the drinking of the fish. However, further investigation is needed to study the relationship between kinins level and drinking response in seabream.

With such a novel observation on the bimodal dipsogenic behaviors of seabream in different salinities, the classical RAS model in fish may need some revision. As suggested by Tsuchida and Takei (1999), the effect of ACE inhibitors is not due to the inhibition of RAS, but to the activation of KKS. Studies using angiotensin analogs such as salarasin ([Sar^1,Ala^8] Ang II) found that such analogs were not effective in fishes (Nishimura *et al.*, 1978). However, such results have been underestimated until recently when Takei and Tsuchida (2000) found that lowering of Ang II level by antibodies did not affect the drinking rate and blood pressure in seawater eel. Such misunderstanding might be due to the generalization in mammalian physiology to other vertebrates. There is a belief that those happen in mammals should also be true in other vertebrates. However, those ACE inhibitors identified in mammals are no longer reliable for the assessment of the RAS in fishes.

3.4.2 Angiotensin converting enzyme activity

The relative ACE activities found in seabream gill tissues at various salinities are different. As the abundance of ACE in long term adaptation may provide us a hint of the status of RAS inside the body. It was shown that the amount of ACE in 6 ppt seabream was the lowest. Upon increasing salinities, ACE became more abundant and ACE activity was the highest in seawater. This may reflect that there is a need for the fish to maintain a minimum level of circulating Ang II level in various salinities. Beside plasma renin activity, brachial ACE activity is a bottleneck of generation of Ang II in plasma. Elevation in ACE activity in seawater means that a larger amount of circulating Ang II is needed. However, ACE has 2 functions (see section 3.4.3), one is to convert Ang I to Ang II, the other is to breakdown active kinins. An increase in ACE in fish gill may also mean a lowering of plasma kinin level. While kinins may inhibit drinking in fish, a lowering of ACE might also be a sign of increase in plasma kinin levels, which may counteract the dipsogenic effect of Ang II. One of the strange phenomena observed in ACE activity in seabream is that the ACE activity in 50 ppt fish is lower than that of 33 ppt fish. One would expect that the ACE activity and also the drinking rate of 50 ppt fish should be the highest among all the experimental salinities. In contrast, 50 ppt fish did not drink more and even had a lower ACE activity. This may be related to elevation of kinin level by lowering of ACE activity in 50 ppt seabream. It is suggested that in 50 ppt medium,

the seabream shifts the status of RAS and KKS to a new homeostatic level by the altering the ACE activity. However, as the plasma levels of angiotensin and kinins are not measured in the present study, no direct evidence could be provided to support the "antagonistic" effect of angiotensin and kinin in fish.

3.4.3 Blood pressure

The ACE inhibitor captopril was found to lower the blood pressure of both 33 ppt and 6 ppt seabream significantly, indicating that RAS may play a part in maintaining the basal blood pressure of the fish. This phenomenon is partly comparable to those found in eel and trout in which captopril only lowered the blood pressure under seawater conditions. However, even under captopril inhibition, the blood pressure of seabream did not change significantly when they are abruptly transferred from 33 ppt to 6 ppt. This suggested that other than RAS, the blood pressure is tightly regulated by the seabream during the period of osmotic challenge.

Captopril has a less significant inhibitory effect on the blood pressure of 6 ppt seabream, suggesting RAS is at a less activated state in hyposmotic medium when compared with the hyperosmotic one. This is also supported by the results of drinking rate in seabream. It was found that 6 ppt fish drank slower than that of 33 ppt fish. Similar results were found when the captopril-inhibited 6 ppt fish were abruptly transferred from 6 to 33 ppt when the blood pressure did not alter

significantly. This also showed that a rapid effector was present in seabream to cope with immediate osmotic challenge to maintain the blood volume and pressure. The blood pressure of seabream appears to be partly affected by, but not tightly regulated by RAS. Alternative perspective concerning the effect of ACE inhibition on blood pressure may lead to more confusing situation. As mentioned above, the kinins level should rise under ACE inhibition. Therefore, the action of increasing kinins level in plasma should not be neglected in the consideration of the blood pressure maintenance in fish as kinins were also found to be vasopressive in eels (Takei, 2000).

Angiotensin II injection elicits an increase in blood pressure of seabream significantly. After injection, the blood pressure return to resting value within 15 min, which indicates that the degradation of the peptide is rapid. Moreover, the vasopressive response produced by Ang II is also rapid. Therefore, the RAS in seabream, like other teleosts, may serve as an anti-drop regulator to maintain the blood pressure. Furthermore, when the captopril-blocked 6 ppt seabream was abruptly transferred to 33 ppt, the blood pressure increased and it took longer for the blood pressure to return to resting level compared with the "blank" 6 ppt fish. A possible reason for such observation was the increase production of kinins, which was found to be vasopressive in eel, but the degradation of kinins was blocked by captopril. However, such explanation was still a hypothesis, further investigation are need to

study the role of angiotensin and kinin, and how they affect the vascular system of the fish.

Through a phylogenetic perspective, the RAS was very conserved from fish to mammals. However, several differences should also be noted through evolution. When vertebrates invaded the terrestrial environment, the RAS was modified to give rise to the aldosterone system. This resulted in a shift in the mode of action of RAS from a volume regulator to an endocrine system concerning salt conservation. On the other hand, the evolution of KKS was little known. The completely opposite effects of kinins in the cardiovascular responses in fishes and mammals indicated that the KKS, at least, underwent different modifications in the 2 lines during evolution. Moreover, in the view of origin, KKS seems to evolve even earlier than RAS, as the Michaelis-Menten constant (K_m) for bradykinin is lower than for Ang I (Ryan, 1983). Therefore, it seems that there is a large missing link between the interaction of RAS and KKS within the classes and across the broad spectrum of animal phylogeny.

Chapter 4

General conclusion

Silver seabream was found to be a "physiologically euryhaline" species as it can survive in various salinities with different but stable physiological and metabolic strategies. Some cardiovascular parameters, such as blood volume and blood pressure, are well maintained no matter the fish is chronically adapted to specific salinities, or abruptly transferred to extreme salinities. An emergency mechanism for regulating the homeostasis during abrupt osmotic challenge must be present in seabream. Classical perspective that the fish percepts osmotic changes by indicators such as blood volume and pressure is not true in seabream as the fish responses before those indicators changes. Respiratory characteristics such as oxygen dissociation curves, hemoglobin composition and blood oxygen levels also show high level of similarities between fish adapted to extreme osmotic environments. Regulation of drinking rate, which indicates the importance of osmoregulation in various salinities, is highly related to the external environment and the responses are rapid. The fish died soon in non-ionic hypertonic medium (1 M sucrose), a phenomenon suggested that ionic inequilibrium may has greater effects than the volume and pressure disturbance. Therefore, it is more likely that seabream sense salinity changes through ionic composition of the external environment and this efficient perception on

ions allows the fish to response more rapidly to reorganize the body structure and function. These demonstrated that silver seabream, although is a true marine fish, is also a good model in the study of piscine euryhalinity. Moreover, the euryhalinity of seabream, to some extent, is even higher than those well-known euryhaline species such as eel. The results of stability in blood volume and blood pressure under abrupt osmotic challenge can be cited as examples.

Studies in teleost fish showed that RAS plays a significant role in osmoregulation, especially in seawater adaptation. This system exhibits a wide spectrum of osmoregulatory effects in various organs such as gill, intestine, kidney, etc. Classically, RAS has been thought to be highly significant in seawater inhabitation. This is supported by the highest ACE activity found in seawater seabream compared to the other salinities. In the present study, manipulation of RAS using ACE inhibitor captopril was found to be able to alter the blood pressure and drinking rate of silver seabream. Mostly, the results are comparable to those obtained from eel and trout. However, injection of Ang II has an anti-dipsogenic effect in seawater seabream. Classically, Ang II is a dipsogen in hyposmotic seabream but it also lowers the drinking rate of seawater seabream, showing a novel perspective of RAS in fish. As suggested by Takei (2000), investigators have been too dependent on ACE inhibitor for the inhibition of RAS in fish. ACE inhibition not only lowers

endogenous Ang II levels, but also leads to accumulation of active kinins of the KKS, an endocrine system that has been neglected for decades. Increasing studies demonstrated that many of the effects that classically are recognized as a result of lowering of Ang II by ACE inhibitors are actually caused by increment of circulating kinins. Therefore, although seabream possesses a bimodal relationship between drinking rate and RAS in different salinities, it might also be a good model in further investigation in the relationship between RAS, KKS and dipsogenic behaviors in fish.

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